University of Alberta

Molecular Biodiversity of Phytoplasmas

by

Keri Wang ©

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Molecular Biodiversity of Phytoplasmas** submitted by **Keri Wang** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Plant Science.**

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ABSTRACT

A new, more efficient procedure based on heteroduplex mobility assay (HMA) was developed for studies of molecular biodiversity of phytoplasmas. By analyzing the artificially introduced substitutions and insertion/deletions into the 16S rRNA gene of aster yellows phytoplasma, HMA was shown to be capable of detecting a single base-pair deletion/insertion or a single two-base-pair substitution in a 520 base-pair DNA fragment. A single base-pair substitution was differentiated by HMA when a suitable reference was used. Heteroduplex mobilities were affected by base number and base composition in mismatches or gaps and were proportional to the degree of DNA divergence. Gaps caused greater retardation in heteroduplex mobility than did mismatches. HMA was also highly sensitive in detecting mixed infections of phytoplasmas.

The partial and full 16S rRNA, the 16/23S spacer region, and the large DNA fragment comprising the full 16S rRNA and 16/23S spacer region amplified from 24 representative phytoplasmas were subjected to HMA analysis. Different groups were clearly discerned in HMA profiles and fully agreed with those derived from DNA sequencing analysis. The 16/23S spacer region was found to be a suitable sequence for using HMA in the classification of phytoplasmas with aster yellows phytoplasma isolate 27 (AY27) as a reference.

A comparative study was conducted to evaluate RFLP and HMA for differentiating closely-related phytoplasmas by analyzing both the 16S rRNA gene and the 16/23S spacer regions amplified from phytoplasma strains of clover proliferation (CP), alfalfa witches'-broom (AWB), and potato witches'-broom (PWB). Two subgroups among each of the phytoplasmas CP, AWB, and PWB were identified by HMA but not by RFLP. A

total of 62 phytoplasma isolates were identified and classified into five major groups by HMA analysis of the 16/23S spacer region. More genetic diversity of phytoplasmas was observed than in previous studies. Twenty-five phytoplasma diseases were reported and identified for the first time in Canada. Phytoplasmas in subgroups I-A and I-B were prevalent in Alberta and more phytoplasmas were found in subgroup I-A than in subgroup I-B. These studies have indicated that HMA is a reliable, rapid, and accurate method for the detection and estimation of the genetic divergence of phytoplasmas when other methods such as RFLP analysis are not readily applicable.

The secondary structure model of the 16S rRNA gene of AY phytoplasma was constructed and the highly variable sections were identified among phytoplasmas in ten major groups. The tRNA^{Ile} located in the 16/23S spacer region was found to evolve slowly. Inferences about the molecular evolution of phytoplasmas were made from a phylogenetic tree with a high confidence level constructed by analyzing the nucleotide sequences of the 16S rRNA gene and the 16/23S spacer region of 24 phytoplasmas. This study suggested that phytoplasmas might have evolved into two branches with different genome sizes from their ancestor and that phytoplasmas in the CP group likely originated from the AP group, with the WX group acting as a possible bridge.

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List of Abbreviations

AAP	Acholeplasma-Anaeroplasma-Phytoplasma
AFLP	Amplified fragment length polymorphism
AP	Apple proliferation
AshY	Ash yellows
AspY	Asparagus yellows
AT	Apply proliferation
AWB	Alfalfa witches'-broom
AY	Aster yellows
AYA	Aster yellows (apricot isolate)
BAC	Bacterial artificial chromosome
BHAY	Belgian hydrangea aster yellows
BLL	Brinjal little leaf
bp	Basepair
BVK	Phytoplasma from leafhopper *Psammotettix cephalotes*
BWB	Bamboo witches'-broom
CalY	Callistephus yellows
CanY	Canola yellows
CaPY	California poppy yellows
CarY	Carrot yellows
cDNA	Complementary DNA
CelP	Celery proliferation
ChAY	China aster yellows
CJWB	Chinese jujube witches'-broom
ClY	Clarkia yellows
CorY	Coreopsis yellows
CP	Clover proliferation
CPaWB	Chinese paulounia witches'-broom
CPh	Clover phyllody
CPY	Cotton poplar yellows

CWB	Chokecherry witches'-broom
CX	Canadian X disease
CYE	Clover yellow edge
DahY	Dahlia yellows
DGGE	Denaturing gradient gel electrophoresis
DicY	Dictamnu albus yellows
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylene diaminetetraacetic acid, disodium salt
ESFY	European stone fruit yellows
EVY	Echium vulgare yellows
EY	Elm yellows
FBP	Faba bean phyllody
FD	Flavescence dorée
FFY	Fever few yellows
FHAY	French hydrangea aster yellows
FMGY	French marigold yellows
GIY	Scabiosa yellows (Giant Imperial)
HMA	Heteroduplex mobility assay
IAWB	Italian alfalfa witches'-broom
IOM	International Organization for Mycoplasmology
JJWB	Japanese jujube witches'-broom
JPaWB	Japanese paulownia witches'-broom
kb	Kilobase
KJWB	Korean jujube witches'-broom
KPaWB	Korean paulownia witches'-broom
MGY	Marigold yellows
MLO	Mycoplasma-like organism
MonY	Monarda yellows
mtDNA	Mitochondrion DNA
ParY	Parsley yellows
PCR	Polymerase chain reaction

PCWB	Pincherry witches'broom
PCY	Purple coneflower yellows
PD	Pear decline
PEP	Picris echioides phyllody
PepY	Pepper yellows
PetY	Petunia yellows
PMGY	Pot marigold yellows
PPT	Potato purple top
PWB	Potato witches'-broom
PYLR	Peach yellow leaf roll
PyrY	Pyrethrum yellows
QALY	Queen Anne's lace yellows
RAPD	Randomly amplified polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal RNA
RudY	Rudbeckia yellows
RYD	Rice yellow dwarf
ScaY	Scabiosa yellows
SCWL	Sugarcane white leaf
SEM	Spiroplasma-Entomoplasma-Mycoplasma
SFY	Straw flower yellows
ShY	Shell flower yellows
SPLL	Sweet potato little leaf
SpY	Spinach yellows
SRDY	Swan river daisy yellows
STOL	Stolbur
STOL	Stolbur of pepper
SUNH	Sunn hemp witches'-broom
TagY	Tagetes yellows
TBB	Tomato big bud

TE	10mM Tris·HCl, 0.1mM EDTA, pH 8.0
TPP	Tower poplar yellows
$\mathrm{tRNA}^{\mathrm{Ile}}$	Isoleucine transfer RNA
TWB	Tsuwabuki witches'-broom
U	Unit
UV	Ultraviolet
VinY	Vinca yellows
VK	Grapevine yellows
VNTR	Variable number tandem repeat
VP	Vinca phyllody
WEY	Winged everlasting yellows
WWB	Willow witches'-broom
WX	Western X disease
YAC	Yeast artificial chromosome
ZinY	Zinnia yellows

I

Literature Review

1.1 Introduction

Phytoplasmas, formerly termed mycoplasma-like organisms (MLOs), are associated with diseases of more than three hundred plant species important to agriculture, horticulture and forestry (McCoy, *et al.*, 1989). The symptoms are characterized by flower malformation, virescence (greening), phyllody, proliferation or witches'-broom, yellowing, and/or decline. The first phytoplasma-associated disease, aster yellows (AY), was described as early as 1926 (Kunkel, 1926) when it was believed to be caused by a virus.

Phytoplasmas were discovered to be associated with plant diseases by observation of microorganisms, morphologically and ultrastructurally resembling mycoplasmas, in the phloem sieve elements of plants affected by mulberry dwarf (MD), potato witches'-broom (PWB), AY and paulownia witches'-broom (PaWB) by electron microscopy in 1967 (Doi *et al.*, 1967). Due to this similarity in morphology and ultrastructure to animal mycoplasmas (members of the class *Mollicutes*), these microorganisms were called MLOs. However, with the advent of molecular biology, analyses of conserved genes such as the 16S rRNA genes, the 16S ribosomal protein genes, and the 16/23S spacer sequences have indicated that plant pathogenic MLOs are significantly different from animal mycoplasmas. Therefore, MLOs were renamed "phytoplasmas" at the 10th International Congress of the International Organization for Mycoplasmology (IOM) in 1994.

Although the inability so far to culture phytoplasmas continuously in cell-free media has hindered research on their characterization and classification, much progress in the area of phylogeny and taxonomy of phytoplasmas has been made since the development of molecular-based tools such as mono or polyclonal antibodies (Chen *et al.*, 1994; Clark *et al.*, 1989; Fos *et al.*, 1992), cloned phytoplasma DNA probes (Chen *et al.*, 1992; Daire *et al.*, 1992; Deng and Hiruki, 1990a, b, 1991b; Kirkpatrick *et al.*, 1987; Ko and Lin, 1994), phytoplasma-specific universal or phytoplasma group-specific polymerase chain reaction (PCR) (Deng and Hiruki, 1990b, 1991a; Davis and Lee, 1993; Gundersen and Lee, 1996; Jaraush *et al.*, 1994; Lee *et al.*, 1993, 1994; Lim and Sears, 1992; Smart *et al.*, 1996), restriction fragment length polymorphism (RFLP) (Ahrens and Seemüller, 1992; Deng and Hiruki, 1991b; Lee *et al.*, 1993, 1998b; Gundersen *et al.*, 1996), and DNA sequencing (Namba *et al.*, 1993; Gundersen *et al*, 1994; Seemüller *et al.*, 1994; Kirkpartick *et al.*, 1994). Numerous new phytoplasma strains have been identified in the last decade. The wealth of molecular data on phytoplasmas has revealed that phytoplasmas are more diverse than previously thought. Twenty major phylogenetic groups have been classified using DNA-based and/or serological methods (Seemüller *et al.*, 1998).

Analysis of genetic variation in natural populations of microorganisms is of importance not only because it tells us about the variability within a single species, but also because it tells us about the mechanisms for genetic exchange and rearrangement including conjugation, transduction and transformation, plasmids, phages, and transposable elements (Young, 1989). Because of asexual reproduction in bacteria, these mechanisms play a major role in species diversity. Therefore, studies of genetic variation in natural populations of phytoplasmas are of great interest and may help understanding why some phytoplasmas are highly variable, while others appear to be much more stable.

Although phytoplasmas are genetically different from mycoplasmas on the basis of molecular analyses of the highly-conserved genes, and they have different genotypic and biological characteristics such as their natural hosts and plasma membrane compositions, they all belong to the class *Mollicutes* and share certain genotypic characteristics such as low G+C contents of their genomes and small genome sizes (Sears and Kirkpatrick, 1994). In addition, as a result of detailed studies, great progress has been made on the broad aspects of mycoplasmas such as molecular biology, genetics, and interaction between mycoplasmas and hosts (Razin *et al.*, 1998). Understanding this impressive information on mycoplasmas will be useful in studying phytoplasmas. Therefore, some reports on mycoplasmas also are included in this review.

1.2 Genetic Basis of Biodiversity

1.2.1 The Definition of Biodiversity

Biodiversity is defined as the diversity of life in all its forms, and at all levels of organization (Hunter, 1996). The structure of biodiversity is indicated in this definition. First, "all its forms" means that biodiversity includes plants, animals, fungi, bacteria, and other microorganisms. Second, "all levels of organization" indicates that biodiversity refers to the diversity of genes and ecosystems as well as species diversity. However, sometimes ecological and evolutionary functions or processes are also included in definitions of biodiversity. The Wildlife Society (1993) defines biodiversity as "the richness, abundance, and variability of plant and animal species and communities and the ecological processes that link them with one another and with soil, air, and water." Biodiversity includes species diversity, ecosystem diversity, and genetic diversity. Only genetic diversity will be examined in this review.

1.2.2 The Sources of Genetic Diversity

Genetic diversity classically refers to the extensive genetic variation within populations containing localized groups of individuals belonging to the same species for which there are no restrictions to random mating among its members (Hunter, 1996). The numerous genes in a population are called the population's gene pool. It consists of all alleles at all gene loci in all individuals of the population. As early as 1908, it was discovered that in the absence of disruptive factors, the frequencies of different genotypes in a population will reach an equilibrium and will remain stable from generation to generation (Hardy, 1908; Weinberg, 1908). The Hardy-Weinberg principle applies to an idealized population of sexually reproducing organisms that is not evolving. It must be considered under the following five conditions: a) infinitely large population; b) isolation from any other populations of the same species; c) no net mutations; d) random mating; e) equal viability, fertility, and mating ability of all genotypes (no natural selection). However, a natural population never meets all of these conditions. Any factor departing from one of the five conditions will cause the allele and genotype frequencies to change, resulting in evolution.

1.2.2.1 Genetic Drift

Natural populations are often finite and small in size. Genetic drift refers to the changes of allele frequencies in the gene pool of a small population due to chance (Campbell, 1987). Two situations can lead to genetic drift. One is the population bottleneck effect that can have important evolutionary consequences. This effect occurs when a normally large population is drastically reduced in size because of unfavorable conditions. Even though the population may eventually recover, genetic drift during the bottleneck can alter the relative abundance of alleles. The frequency of the alleles would depend on the number of survivors from the original population.

Another extreme case of genetic drift is the founder effect, whereby a small number of dispersed individuals manage to establish a new population. The most extreme case would be the establishment of a new population by one pregnant animal or a single plant seed. Simply by chance, the allele frequencies at many gene loci are likely to be different from what they were in the original population, and the new assortment will dictate the genotypic character of the new population.

The occurrence of genetic drift in phytoplasmas has been postulated recently (Lee *et al.*, 1998a). Phytoplasma-associated plant diseases are spread primarily by insect vectors belonging to the families *Cicadellidae* (leafhoppers) and *Fulgoroidae* (planthoppers). When the insect vectors carrying phytoplasmas fly or are transported from one place to another over long distances, the insects may voluntarily feed or be forced to feed on nonhost plants in places where no host plants are available. Under certain conditions, the nonhost plants will become infected if they become susceptible to the phytoplasma(s) carried by the visiting vectors. The phytoplasma in this new host plant becomes isolated from the common pool shared by any member of the given phytoplasma group, and the extent of genetic exchange from the strain pool greatly decreases. Since the genetic exchange for this isolated phytoplasma strain is different from the parental strain pool, a phytoplasma will evolve independently from the parent strain. The phytoplasma may be exposed to a new group of insect vectors and begin to establish a new biological cycle. The phytoplasma in the new host may be transmitted by a non-native insect or by vegetative propagation. A new phytoplasma-associated disease may be expected to emerge sometime during the formation of new ecological niches. New vectors, if present, then transmit and spread the phytoplasma. The less diverse phytoplasma groups, such as apple proliferation (AP), may have been formed by this mechanism.

1.2.2.2 Gene Flow (Migration)

In many species, their populations are networks of sub-populations with intermittent migration between neighboring sub-populations. Migration of individuals between populations results in genetic exchange. Gene flow is the physical movement of alleles in and out of populations. It tends to decrease the genetic differentiation among populations that arises through other evolutionary factors (Starr and Taggart, 1989).

Gene flow also may happen in phytoplasmas, although they cannot migrate by themselves for long distances. The long-distance transportation of phytoplasma-infected materials makes gene flow of phytoplasmas possible. Without strict quarantine inspection, phytoplasma-infected materials such as budwood, rootstock, and tubers can be introduced through international exchanges into new geographic regions where native vegetation and vector species are different from the region from which the phytoplasmas were inadvertently transported. If all necessary elements for sustaining the phytoplasma(s) are available, such as susceptible plant species and available vectors, a new ecological niche for the "imported" phytoplasma(s) is created. The newly introduced phytoplasmas have opportunities to interact with indigenous plants, vectors, and phytoplasmas (Lee *et al.*, 1998a).

In nature, certain phytoplasmas share the same insect vectors and host plants. For example, clover can be infected by phytoplasmas of clover phyllody (CPh) (Lee *et al.*, 1992a), clover proliferation (CP) (Chen and Hiruki, 1975), and clover yellow edge (CYE) (Sinha and Benhamou, 1983). In Australia, a leafhopper (*Orosius argentatus*) was reported to transmit phytoplasmas of legume little leaf, tomato big bud (TBB), lucerne witches'-broom, virescence of tobacco, and potato purple top (Grylls, 1979). The overlapping vectors and plant hosts may make it possible for the phytoplasmas to interact with one another and to exchange their genetic information vertically and horizontally among these phytoplasmas in the host pool. As a result, a diverse group of phytoplasmas is formed with continuous and wide genetic variation among its members. For example, the AY group consists of nine distinct subgroups with numerous strains that are distributed in numerous host species worldwide, and the X-diseases (WX) group comprises eight subgroups on at least three continents (Lee *et al.*, 1998b).

1.2.2.3 Mutation

Mutations are changes in genes that give rise to altered forms, most, but not all, of which function less well than the wild-type alleles, while a mutant is a new variant form of a gene (Watson *et al.*, 1987). Mutations may involve base substitution including transition and transversion, insertion or deletion of one or more bases which may result in frame shifting, or rearrangement of a section of DNA including inversion and conversion. Although mutations at a particular gene locus are rare, the cumulative impact of mutations at all loci can be significant. This is because each individual has numerous genes, and many populations have thousands or millions of individuals. Over the long term, mutation is very important to evolution because it is the original source of the genetic variation that serves as a gene pool for natural selection.

Mutations have been accumulating for millions of years. The new alleles that appear by mutation in a given population are not harmful, neutral, or beneficial in themselves; their effects depend on the environment in which their gene products are expressed. However, since the structural, functional, and behavioral traits of an organism already allow it to function well in a particular environmental context, any drastic change is more likely to upset things than to enhance them. Therefore, most mutations with large effects are deleterious, even lethal, to the organisms (Campbell, 1987).

The members of the class *Mollicutes* are characterized by the lack of a cell wall, small genome size (580 to 2,220 kb), low guanine-plus-cytosine (G+C) content (most between 24 and 33 mol %), and unusual nutritional requirements (Razin *et al.*, 1998). Analyses of the 16S rRNA gene sequences for bacteria evolution have indicated that mollicutes evolved from ancestors that were common to gram-positive bacteria - the *Lactobacillus* group - with low G+C contents and a genome size about 4,000 kb (Maniloff, 1992). Therefore, mollicutes are rapidly evolving microorganisms having unusual phenotypes and small genome sizes. The high mutation rate may be related to the reduction in genome size and is responsible for the adaptability of mycoplasmas to changing environments.

Samuelsson and Borén (1992) proposed a model, for the reduction in genome size, which postulated that gene economy was the driving force for the severe reduction in genome size. Economization of DNA content is a characteristic property of all prokaryotes. All bacteria are streamlined organisms that, during their evolution, probably lost a large portion of their original non-informational DNA, such as the introns and other noncoding nucleotide sequences that have been preserved by eukaryotic organisms (Doolittle, 1978, 1980; Orgel and Crick, 1980). Excess DNA is an energy burden, and it is conceivable that under such conditions where the supply of metabolic energy is severely limited, small genomes that can be exactly and efficiently replicated will have a selective advantage. The organisms from which the mycoplasmas evolved may have suffered occasionally from such a lack of energy supply. The loss of genetic material was tolerable in mycoplasmas because they were parasitic in nature and capable of acquiring many complex organic molecules through their host organisms. Many genes involved in biosynthetic pathways could, therefore, be deleted (Samuelsson and Borén, 1992).

A high mutation rate, i.e. the number of mutations per base pair and cell generation, also is related to the reduction in genome size (Drake, 1974; Maniloff, 1983). While a high mutation rate is advantageous from an evolutionary point of view, the maximum level of errors must be restricted to avoid deleterious effects (Samuelsson and Borén, 1992). The total number of errors introduced into a genome is roughly proportional to the size of genome. Therefore, an organism with a smaller genome can tolerate a comparatively high mutation rate. Consequently, as genetic materials deleted from chromosomes increase during the evolution of mycoplasmas, it is likely that mutation rates gradually increase, finally reaching the level observed in mycoplasmas today (Maniloff, 1978; Woese *et al.*, 1980).

It has been proposed that the low G+C content of the DNA of mollicutes is due to an AT-biased mutation pressure, perhaps caused by variations in the amounts or activities of enzymes involved in DNA replication and repair (Williams and Pollack, 1988). Mycoplasmas are known to lack some of DNA repair mechanisms such as dark repair or photoreactivation (Ghosh *et al.*, 1977; Labarère 1992). The DNA replication apparatus appears to be simplified in some species of mycoplasmas in the sense that there seems to be only one DNA polymerase in these organisms (Maurel *et al.*, 1989; Mills *et al.*, 1977). Studies of genetic maps in mycoplasmas have revealed the absence of many genes, including such major genes involved in DNA replication of *M. genitalium* and *M. pneumoniae* as initiation, elongation, and termination of replication (Himmelreich *et al.*, 1996, 1997). All of these systems may be relevant to the increased rate of mutation observed in mollicutes.

The number of tRNA genes in mollicutes is also reduced to a minimum, with very few gene duplicates. Only 30 and 33 tRNA genes have been found in *M. capricolum* and *M. pneumoniae*, respectively (Andachi, *et al.*, 1989; Simoneau *et al.*, 1993). It is the smallest number among all known genetic systems, except for mitochondria. Accordingly, the number of anticodons in *M. capricolum* is only 28 and that in *M. pneumoniae* is only 32, which is close to the theoretically essential minimum of 23 anticodons needed to translate all 61 amino acids codons according to the wobble rules (Andersson and Kurland, 1995; Bové, 1993). Thus, the limited coding space of the mycoplasmal genome has led to a paucity of tRNA genes achieved by the use of a simplified decoding method (Simoneau *et al.*, 1993). The mycoplasmal tRNAs contain significantly fewer modified nucleosides than do other bacterial tRNAs, economizing in genes for enzymes involved in tRNA post-transcriptional nucleoside modification. Thus, only 13 types of modified nucleosides were found in the total tRNAs of *M. capricolum*, compared to 23 types in *E. coli* (Andachi, *et al.*, 1989; Bové, 1993).

1.2.2.4 Nonrandom Mating

Mating is a characteristic of sexually reproducing organisms. Random mating is one of the conditions needed to maintain the Hardy-Weinberg equilibrium in a population. However, individuals usually mate more often with close neighbors than with more distant members of the population (inbreeding), or mate with the partners that are like themselves in certain phenotypic characters (assortative mating) (Hartl and Clark, 1989). Nonrandom mating increases the number of gene loci in the population that are homozygous. Any change in a population's inbreeding or assortative mating behavior will shift the frequencies of different genotypes. Thus, nonrandom mating can cause a population to evolve.

Mollicutes, including phytoplasmas, are prokaryotes subject to asexual reproduction. Therefore, mating behavior does not exist in phytoplasmas. However, the cells may exchange their genetic information by conjugation, transduction and transformation, plasmids, phages, and transposable elements if they contact each other during multiplication (Young, 1989). Genetic recombination plays a major role in the evolution of prokaryotes. However, little is known about phytoplasmas in this respect.

1.2.2.5 Natural Selection

The principle of natural selection was first stated by Charles Darwin in 1859. Those individuals that possess characters beneficial for the competitive existence will be more likely to survive than those without them. The survivors will pass these advantageous characters onto their offspring, so that successful variations will be transmitted to future generations. There are three major modes of natural selection (Starr and Taggart, 1989). Stabilizing selection favors intermediate forms of a trait and operates against extreme forms; hence, the frequencies of alleles representing the extreme forms decrease. Directional selection shifts the phenotypic characters of the population as a whole, either in response to a directional change in the environment, or in response to a new environment. Therefore, the allelic frequencies underlying the range of phenotypes move in a steady, consistent direction. Disruptive selection favors extreme forms of a trait and operates against intermediate forms. As a result, the frequencies of alleles that represent the extreme forms increase.

The low G+C content and small genome size of mollicutes are suggested to be the results of a high mutation rate and directional natural selection (Samuelsson and Borén, 1992). The organisms from which the mollicutes evolved may have suffered from a lack of energy supply from time to time. To adapt to changing environmental conditions and to utilize limited energy economically, many genes involved in biosynthetic pathways could be eliminated. This could have been accomplished by a high rate of mutational events. As a consequence, a large amount of genetic material was lost, although this loss was not a selective advantage in itself. The remaining genes are all essential for survival. Therefore mollicutes cannot delete more genes.

The probability of AT bias in mollicutes is dependent on the particular selection pressure. The noncoding sequences contain high frequencies of A·T base pairs (close to 80%), whereas coding regions require a high content of G·C base pairs (close to 50%)

(Muto and Osawa, 1987; Osawa *et al.*, 1987). During the evolution of mollicutes, all genes that were not essential for survival would be sensitive to the AT bias, so that a majority of G·C base pairs in these genes were replaced by A·T base pairs, thus converting a coding gene to a noncoding region with a predominance of A·T base pairs. Such regions in the chromosome might have had some specific properties that facilitated their elimination from the genome.

1.2.3 The Importance of Genetic Diversity

The wealth of genetic diversity is very important in species evolution, fitness and utilitarian values (Hunter, 1996). All species have to adapt to changing environments if they are to survive. Species with greater genetic diversity are more likely to be able to evolve in response to an ever-changing environment than those with less diversity. Natural selection is genetically based on variability in the fitness of individuals; that is, individuals with a greater ability to adapt to a new environment will better be able to survive and reproduce in this environment than individuals with less ability. If every individual were genetically identical and only chance determined which ones left progeny, then populations would change very slowly and could become extinct if there were dramatic changes in the environment (Hartl and Clark, 1989; Hunter, 1996). Environments change through space as well as time. A species with greater genetic diversity is more likely to establish itself in a wider range of environments than a species with limited genetic diversity. This also helps the species survive in changing environments. The genetic diversity of some wild populations is also important to plant and animal breeders because wild relatives of domestic species are a significant source of genetic material.

Populations that lack genetic diversity may experience problems even in static environments. In plant pathology, the cultivation of genetically uniform varieties over several million acres presents a huge opportunity for the development of devastating disease epidemics (Agrios, 1988). Many devastating epidemics resulted from the cultivation of varieties with genetic uniformity over a large area. Huge economic losses were caused in each case. For example, the widespread use of corn hybrids containing the

Texas male-sterile cytoplasm made it possible for a previously unimportant race of the fungus *Helminthosporium maydis* to spread widely, and consequently the southern corn leaf blight destroyed more than a billion dollars worth of corn in the United States in a single year (Ullstrup, 1972).

Some phytoplasmas also have brought their host plants to near extinction. For example, the lethal yellowing-type diseases of coconut palm, caused by phytoplasmas, have been endemic in several countries of the western Caribbean region and central America (Ashburner *et al.*, 1996; Harrison *et al.*, 1992; Howard and Barrant, 1989). Millions of susceptible tall-type coconut palms have been killed during the last three decades and have been virtually eliminated from many locales. These diseases are the most significant threat to world coconut palm production (Harries, 1978). Therefore, the wild population should be studied for the breeding of disease-resistant varieties of coconut plants.

1.3 Molecular Study of Genetic Diversity

Molecular techniques permit objective quantification of genetic diversity. Such measurements of genetic diversity can help define priorities, reduce costs, and optimize management decisions. Molecular analysis provides an important tool for the characterization of biodiversity.

1.3.1 Allozymes

Since the 1960s, allozymes, isozymes produced by orthologous genes differing in composition by one or more amino acids due to allelic differences, have been frequently used in studies of genetic diversity (Soltis and Soltis, 1990).

Data on allozyme variation have revolutionized the understanding of population differentiation and the structure of genetic diversity in natural populations (Hamrick, 1990; Weeden and Wendel, 1990), especially in organisms such as plants that may show high levels of allozyme polymorphism. In mollicutes studies, understanding of amino acid substitutions in mollicutes enzymes would be useful in taxonomic and evolutionary studies (O'Brien *et al.*, 1983). Analysis of allozymes has led to the construction of phylogenetic trees at the genus level which are the same as the dendrograms developed from sequencing data (Tully *et al.*, 1993; Weisburg *et al.*, 1989; Woese *et al.*, 1980). Therefore, an expressible enzymatic function may provide a more practical and representative basis for a useful taxonomy and perhaps even a phylogeny (Pollack *et al.*, 1997).

One advantage of allozyme analysis is its relatively low cost. It can handle large sample sizes and uses simple, relatively unsophisticated equipment. Another advantage is that allozyme markers are co-dominant. Both alleles in a diploid organism are clearly identifiable, and heterozygotes can be discriminated from homozygotes, which is a prerequisite for the estimation of allele frequencies in population genetic studies. However, allozyme analysis underestimates the amount of genetic diversity because of the redundancy of the genetic code (Weising *et al.*, 1995; Liu and Furnier, 1993; Peakall *et al.*, 1995). Use of allozymes also restricts the study to those genes that code for water-insoluble and cell structure-bound enzymes. In addition, it is well-known that allozyme patterns can show considerable variation among different stages of growth and development within individual plants (Alvarez and King, 1969).

1.3.2 Microsatellites and Minisatellites

Microsatellites and minisatellites are ideal genetic markers, and would provide the specificity and rapidity of PCR with information of the variable number tandem repeat (VNTR) loci. VNTRs have a rapid turnover rate and are composed of tandemly repeated simple sequences in the non-coding regions of chromosomes. VNTRs are divided into microsatellites, for which the basic repeat unit is two to eight base pairs in length, and minisatellites, which have longer repeats of 11 to 100 base pairs (Nakamura *et al.*, 1987). The most common repeats are dinucleotides of $(CA)_n$, $(CT)_n$, and $(AT)_n$. The formation of long, tandemly repetitive, and polymorphic tracts is due to replication slippage: i.e., strand displacement followed by an out-of-frame pairing of repeated sequences either resulting in a net gain or loss of one or more repeat units (Levinson, 1987; Levinson and Gutman, 1987; Schlötterer and Tautz, 1992). Analysis of plant gene sequences in databases indicates that microsatellites are less abundant than in mammals (Lagercrantz *et al.*, 1993) and should be found on average once every 50 kb throughout the plant genome, thereby making them very useful as genetic markers (Morgante and Olivieri, 1993).

Microsatellites have become popular molecular markers in a variety of applications that require highly polymorphic and locus-specific genetic systems, such as linkage analysis, paternity testing, genetic mapping, population and evolutionary genetics, and gene flow (Lynch, 1990; Wolff *et al.*, 1994; Dib *et al.*, 1996; Nybom and Schall, 1990). Microsatellites have been used recently in a study of molecular evolution of prokaryotes (Field and Wills, 1998). The study indicated that the high microsatellite polymorphism in *Saccharomyces cerevisiae*, and the different distributions of microsatellites in eight prokaryotes and *S. cerevisiae*, resulted from strong mutation pressures and a variety of selective forces. The results suggested that mutation pressure overcomes overall selection for small genome size that will tend to shorten or eliminate unnecessary DNA.

The use of microsatellites involves the development of primers specific to the organism, which is time-consuming, laborious, and expensive. However, it has been found that the primers derived from various species can be used in closely-related species, but not in more distantly-related species (Primmer *et al.*, 1996; Fields and Scribner, 1997). For the dinucleotide repeats, the slippage always occurs during PCR amplification, which makes the interpretation of correct allele sizes difficult.

1.3.3 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) was one of the first methods for genetic fingerprinting. It involves the digestion of genomic DNA with a restriction enzyme, electrophoresis, Southern blotting, and hybridization with appropriate multilocus labeled probes. The probe is a cloned DNA fragment or polymerase chain reaction (PCR)-amplified gene that shares homology with a target sequence and is thus able to hybridize to a corresponding sequence or sequences in the genomic DNA.

The restriction patterns are particularly effective in determining genetic homogeneity or heterogeneity of strains within established mollicutes species (Razin *et al.*, 1983). The rRNA gene probes derived from *Mycoplasma capricolum* made it possible to effectively demonstrate genotypic heterogeneity among strains of species such as *Ureaplasma* *urealyticum* (Razin and Yogev, 1986), *M. hominis* (Christiansen and Andersen, 1988), *A. laidlawii* (Yogev *et al.*, 1988a), *M. capricolum*, *M. mycoides* (Christiansen and Erno 1990), *M. gallisepticum*, and *M. dynoviae* (Kleven *et al.*, 1988; Yogev *et al.*, 1988b). RFLP analysis using cloned DNA probes also further differentiated phytoplasma strains within a given group into different subgroups (Ko and Lin, 1994; Nakashima *et al.*, 1993).

Due to the small size of mollicutes genomes, the number of restriction fragments produced is relatively small, and even with enzymes like *Eco*RI which cut the mycoplasma genome in many sites, the electrophoretic patterns exhibit well-defined bands, often without smears (Bové and Saillard, 1979). RFLP using carefully selected restriction enzymes has made it possible to construct the physical genomic maps for a number of mycoplasma species (Bautsch 1988; Pyle and Finch, 1988; Wenzel and Herrmann, 1988, 1989; Cocks *et al.*, 1989; Colman *et al.*, 1990; Krause and Mawn, 1990; Pyle *et al.*, 1990; Miyata *et al*, 1991; Tigges and Minion, 1994; Gorton *et al.*, 1995). The genetic maps for some mycoplasma species also were established by the identification and localization of genes on the genome, which were normally done by specific cross-hybridization using the conserved genes of the same or related species as DNA probes (Pyle *et al.*, 1990; Ladefoged and Christiansen, 1992; Ye *et al.*, 1992; Peterson *et al.*, 1995). Therefore, a comparison of genetic maps could provide a sound basis for evaluating phylogeny, understanding evolutionary processes such as the acquisition or loss of species-specific features during development, and determining the differences between strains of the same species. It could also help us study other parameters, such as gene placement on the chromosome in correlation with a non random G+C distribution, which is suggested by a nonstatistical distribution of recognition sites for restriction endonucleases (Cocks *et al.*, 1989; Pyle and Finch, 1988), or the conservation of mechanisms regulating the organized expression of genes or operons that have to function in a certain order.

The *in situ* distribution of phytoplasmas is restricted to the phloem sieve elements of plants. Their titers in infected plants are very low (less than 0.1% of total DNA in extracts from woody hosts exhibiting disease symptoms) (Kirkpatrick, 1989) and vary as the disease develops (Deng and Hiruki, 1990a). To date, phytoplasmas cannot be cultured *in* *vitro*. These facts make it very difficult to obtain pure phytoplasma chromosomes from infected plants in sufficient quantity for physical mapping. For this reason, only three physical maps of phytoplasmas have been constructed so far. The first physical map of a phytoplasma was constructed from the chromosome of the western X-disease (WX) phytoplasma in 1996 (Firrao *et al.*, 1996). The WX phytoplasma was transmitted to celery in which the concentration of phytoplasmas was believed to be higher than in other plants. The locations of 20 restriction sites for the enzymes *Sal*I, *Xho*I, *Baa*HII, *Rsr*II, *Sma*I, and *Not*I were mapped on the chromosome, which is circular and comprises approximately 670 kb.

However, the methods used to isolate the intact genomic DNA of WX phytoplasma failed to extract the intact genomic DNA of apple proliferation (AP) phytoplasma (Lauer and Seemüller, 1998). In contrast, AP phytoplasma was transmitted to tobacco (*Nicotiana tabacum* cv. Samsun), and the physical map was successfully constructed (Lauer and Seemüller, 2000). The locations of the two rRNA operons which seem to be present in all phytoplasmas have been determined (Schneider and Seemüller, 1994). Four functional genes of AP phytoplasma, including the genes encoding elongation factors G (*fus*) and Tu (*tuf*), a gene encoding an immunodominant protein, and a putative nitroreductase gene, were placed on the physical map. The map revealed that the two rRNA operons are separated, while *fus* and *tuf* genes are on the same fragment as one of the rRNA operons. The arrangement of these genes (5'-*fus*-*tuf*-3') may be different from culturable mollicutes in the genus *Mycoplasma* in which the *fus* and *tuf* genes are separated (Berg and Seemüller, 1999).

Recently, the physical map of sweet potato little leaf (SPLL) phytoplasma has been constructed (Padovan *et al.*, 2000). Several genes, including two rRNA operons, the linked *fus*/*tuf* genes, and a *gid* gene encoding a glucose-inhibited division protein, were located on the physical map. An inversion of one of the rRNA operons was inferred from the hybridization data. The map suggested that the putative origin of replication of SPLL phytoplasma, *oriC*, is upstream of the location of pH80. These physical maps have provided a framework for characterizing certain genes and expanded our understanding of the genomic diversity and the genetic architecture of phytoplasmas.

1.3.4 PCR (Polymerase Chain Reaction)-RFLP

This technique combines the ability of PCR to amplify a specific DNA fragment (normally a highly-conserved gene) from relatively crude genomic DNA, with the ability of RFLP to discriminate among genotypes based upon the presence or absence of restriction sites within the amplified DNA. This approach has been extensively used in studies of genetic diversity and phylogeny of phytoplasmas. RFLP analysis of the PCR-amplified 16S rRNA genes and ribosomal protein genes has resulted in the identification and classification of a broad range of phytoplasmas. Ahrens and Seemüller (1992) first used this approach to categorize 17 phytoplasma isolates into four distinct groups. A similar RFLP analysis of PCR-amplified phytoplasma 16S rRNA genes established nine distinct 16S ribosomal RNA (16Sr) groups and 14 subgroups (Lee *et al.*, 1993). The phytoplasmas in the AY group and the WX group were further studied by RFLP analysis of both the 16S rRNA genes and ribosome protein genes, and were differentiated into nine subgroups in the AY group and eight subgroups in the WX group (Gunderson *et al.*, 1996). Based on extensive RFLP analysis of the phytoplasma 16S rRNA genes and ribosomal protein genes, 14 major groups and 38 subgroups were classified among all phytoplasmas used in the study (Lee *et al.*, 1998b). At present, RFLP analysis of PCR-amplified ribosomal DNA is a routine method for the differentiation and classification of phytoplasmas.

The results of diversity studies by RFLP, including PCR-RFLP, are highly reproducible among laboratories. The diversity profiles thus generated can be reliably compared. In addition, no sequence-specific information is required and the approach can be applied immediately for diversity screening in a new species, provided suitable primers are available. However, for classic RFLP, a good supply of probes is required. The blotting and hybridization steps are time-consuming and difficult to automate, and sufficient quantities of high quality DNA are required. The use of radioisotopes or fluorescent labels for detection of the bands is technically demanding, time-consuming, and costly.

The major disadvantage of RFLP is that RFLP may fail to detect a large fraction of mutations and polymorphisms since the probability that a base change will alter a restriction site is low. For example, many of the single mutations in the human β-globulin gene that are known to cause thalassaemia do not alter a restriction enzyme cleavage site, and thus cannot be directly detected by the RFLP method (Orkin and Kazazian, 1984). In addition, RFLP may incorrectly increase the phylogenetic distance between closely-related strains, or fail to detect significant differences between two different strains (Seemüller *et al.*, 1994; Kuske and Kirkpatrick, 1992). DNA sequence analyses of the 16S rDNA of more than 20 phytoplasma isolates in previous studies have indicated that the RFLP-based classification of phytoplasmas in certain groups did not fully coincide with the phylogenetic relationships of the organisms (Seemüller *et al.*, 1994; Kuske and Kirkpatrick, 1992). Therefore, different RFLP patterns do not always indicate significant phylogenetic distances.

1.3.5 Heteroduplex Mobility Assay (HMA)

Heteroduplex mobility assay (HMA), originally developed for the detection and estimation of genetic divergence between human immunodeficiency viruses (Delwart *et al.*, 1993), is a new, rapid, accurate and simple screening tool that not only can distinguish between individual strains, but can also permit reliable inferences to be made about phylogenetic relationships among strains of microorganisms. HMA is based on the observation that DNA heteroduplexes formed between sequences have a reduced mobility, which is proportional to their degree of divergence, in polyacrylamide gels, but not in standard agarose gel. Heteroduplexes are generated by base pairing between complementary single strands derived from the different parental duplex molecules during genetic recombination. Unknown DNA sequences can be compared against themselves or standard reference sequences. The DNA sequences of genetically common or rare variants could, therefore, be determined on a selective rather than random basis. In addition, HMA can be used for tracking specific sequence variants within individuals and assisting in establishing epidemiological linkages between individuals (Delwart *et al.*, 1994).

The high sensitivity of HMA for differentiating among closely-related phytoplasma strains has been reported in previous studies (Ceranic-Zagorac and Hiruki, 1996; Wang and Hiruki, 1999, 2000b). HMA is capable of detecting a single base-pair deletion/insertion or differentiating a single base difference in a DNA fragment of about 500 bp (Wang and Hiruki, 1999, 2000b). Closely-related phytoplasma isolates such as tomato stolbur (France) and eastern aster yellows (New York) phytoplasma isolates were distinguished by HMA but not by RFLP (Zhong and Hiruki, 1994b; Ceranic-Zagorac and Hiruki, 1996). Slight but significant differences were detected by HMA between poplar witches'-broom phytoplasma isolates causing severe symptoms in France and less severe symptoms in Germany (Cousin *et al.*, 1998). HMA has been extremely powerful in detecting mutations of phytoplasmas, thus providing an important tool for detecting and estimating the genetic diversity of phytoplasmas when restriction enzyme recognition sequences are not available for RFLP analysis.

HMA also has a high capacity for aiding the identification and classification of phytoplasmas (Zhong and Hiruki, 1994b; Wang and Hiruki, 2000a). Initially, nine phytoplasma isolates from North America, Asia, Africa, Australia and Europe were classified into four groups by HMA (Zhong and Hiruki, 1994b). Twenty-four representative phytoplasma strains were classified into ten major groups. The results were in agreement with those obtained from sequence data, but not necessarily with those from RFLP (Wang and Hiruki, 2000a). More recently, two subgroups were identified by HMA among the closely-related phytoplasmas, including AWB, CP, and PWB (Wang and Hiruki, 2000b) that could not be differentiated previously by DNA-DNA hybridization and RFLP analysis (Deng and Hiruki, 1991b; Lee *et al.*, 1991). Fourteen field phytoplasma isolates from ornamental plants were identified by HMA and classified into two subgroups in the AY group (Wang and Hiruki, 2001b). Therefore, since HMA not only differentiates closely-related phytoplasma isolates with high sensitivity, but also can be used to estimate the degree of DNA divergence, HMA is an ideal means for the study of genetic diversity and classification of phytoplasmas and other organisms.

1.3.6 Random Amplified Polymorphic DNA (RAPD)

The random amplified polymorphic DNA (RAPD) assay was first developed in 1990 (Williams *et al.*, 1990). It uses PCR to amplify DNA fragments from genomes using random primers. The difference between RAPD and standard PCR is that only a single oligonucleotide primer is employed in the former. The primers are 10-mers rich in GC content (at least 50%) so that a primer-DNA hybrid can withstand the temperature at which polymerization takes place (72°C). To obtain an amplified product from only one primer, two identical (or highly similar) target sequences in close vicinity to each other must be present on opposite strands arranged in the opposite direction.

RAPD has been widely used for genetic diversity studies of culturable microorganisms, plants and animals, as well as for the construction of genetic maps in crop plants (Li and Yeh, 2001). In mollicutes studies, however, only limited efforts were made in using RAPD to study genetic diversity. Knox and Timms (1998) used seven arbitrary primers to differentiate the two biovars of *Ureaplasma urealyticum* and to identify 13 subtypes.

The inability to culture phytoplasmas *in vitro* has hampered the isolation of pure phytoplasma DNA, which in turn makes it difficult to utilize some molecular biotechnologies such as RAPD and amplified fragment length polymorphism for diversity studies of phytoplasmas. So far, only a few references are available for differentiation of phytoplasmas by RAPD (Chen *et al.*, 1994; Zhong and Hiruki, 1994a) together with other techniques such as immunology, dot blot hybridization, and RFLP. However, the RAPD markers produced from the host plants or phytoplasmas were questionable since the host plants used in the studies were not from a single clone. Thus, it is possible that the RAPD markers were derived from individual plants instead of the phytoplasmas. The results showed that some markers are unique to certain phytoplasma isolates (Chen *et al.*, 1994). However, the control DNA from healthy plants was not included in the experiment and no duplicate isolates were examined.

The advantages of using RAPD are that there is no requirement for DNA probes, no information of DNA sequences needed for designing specific primers, and no blotting or hybridization steps. The presence or absence of a particular amplification product in different organisms can serve as a highly informative character for the evaluation of genetic diversity and relatedness. Therefore, the procedure is quick, simple, and efficient.

However, RAPD markers are dominant and heterozygotes cannot be detected, which is a problem in population studies since the allele frequencies cannot be determined accurately (Schierenbeck *et al.*, 1997). Therefore, the value of RAPD markers in diversity studies has been questioned, although the potential skewing effect can be somewhat reduced by examining a large number of polymorphic RAPD bands and increasing sample size (Lynch and Milligan, 1994). In addition, RAPD is sensitive to different reaction conditions, particularly DNA quality and PCR temperature profiles (Muralidharan and Wakeland, 1993). This is due to the fact that the primers have no specific target and thus many sites in the genome are potential targets, resulting in low reproducibility among different laboratories (Karp *et al.*, 1996). Furthermore, RAPD may produce different banding patterns with DNAs extracted from different tissues or different life stages of the same plant. Lastly, the existence of identical bands does not ensure 100% homology, since the identity and the sequence context of a particular amplification product is unknown.

1.3.7 Amplified Fragment Length Polymorphism (AFLP)

The amplified fragment length polymorphism (AFLP) technique combines the reliability of restriction digestion of genomic DNA with the benefits of PCR-based assays by ligating primer recognition sequences (adapters) to the restricted DNA (Zabeau and Vos, 1993; Vos *et al.*, 1995). Two types of polymorphisms are detected with AFLP: a) substitutions in the restriction sites or in the selective nucleotides of the primers, which result in loss of the band, and b) insertions/deletions within the restriction fragment, which results in different sized bands.

AFLP has several advantages over RFLP and RAPD for fingerprinting of a whole genome. AFLP banding patterns are not particularly sensitive to the initial concentration of template DNA (Vos *et al.*, 1995; Zhu *et al.*, 1998). AFLP bands tend to behave similarly when reaction conditions are varied (Zhu *et al.*, 1998). The insensitivity of AFLP to DNA mixtures decreases the potential for erroneous scoring due to sample contamination and for detecting polymorphism in bulked DNA samples (Zhu *et al.*, 1998). The AFLP approach provides 10 to 100 times more markers, on average, than RFLP or RAPD (Lin and Kuo, 1995; Sharma *et al.*, 1996).

The AFLP procedure is particularly suitable for mapping and fingerprinting. It has been widely applied in gene isolation (Thomas *et al.*, 1995), quantitative trait loci analysis (Quarrie *et al.*, 1997), as well as in other genetic and physical mapping studies (Becker *et al.*, 1995; Mackill *et al.*, 1996; Maughan *et al.*, 1996). As a DNA fingerprinting technique, AFLP is efficient in revealing diversity at and below the species level, and provides an effective means for covering a large portion of the genome in a single assay (Hill *et al.*, 1996). The wide coverage is important for the use of AFLP markers in biodiversity studies (Parsons *et al.*, 1997). The speed, reliability, and versatility of AFLP markers in genetic diversity studies make it a valuable tool.

Recently, AFLP also has been employed to study the genetic diversity of mollicutes. AFLP fingerprints of 38 strains of *M. capricolum* derived from different countries in Africa and the Middle East revealed the existence of two major clusters that are equivalent to the two evolutionary lines of the organism found by 16S rDNA analysis (Kokotovic *et al.*, 2000). As well, AFLP was capable of discriminating among the strains at species and intraspecies levels. Subtle intraspecies genomic differences were detected among 50 strains of all the *Mycoplasma* species. The extent of polymorphism varied markedly among the analyzed mycoplasmas with pattern similarity levels varying from 61.7% in *M. dispar* strains to 95.9% in *M. genitalium* strains (Kokotovic *et al.*, 1999).

1.3.8 DNA Sequencing

DNA sequencing is the most important method for characterizing genes, exploring the mechanisms of transcription, and studying gene arrangements and mutations. The genomes or mitochondrial DNA of several plants, animals, insects and microorganisms have been completely sequenced in worldwide collaborative projects. So far, the chromosomes of several mycoplasmas have been completely sequenced (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996). The first report of the complete sequence of the *M. genitalium* genome was jointly published in a co-laboratory project (Fraser *et al.*, 1995). This mycoplasma carries the smallest genome known so far for a self-replicating organism, a genome of only 580 kb.

Complete DNA sequencing of highly conserved genes such as the 16S rRNA genes is an important method for studying genetic diversity, molecular evolution, and for the construction of phylogenic trees of prokaryotes. By comparing the 16S rRNA gene sequences among numerous mollicutes and several walled prokaryotes, mollicutes have been determined to arise from the gram-positive bacterial ancestor of the lactobacillus group (Weisburg *et al.*, 1989; Maniloff, 1992). On the basis of 16S rRNA sequence analyses, phytoplasmas have been classified as a distinct monophyletic clade within mollicutes, and are closely related to acholeplasmas (Lim and Sears, 1989, 1992; Kuske and Kirkpatrick 1992). Twelve groups were identified as distinct subclades of the phytoplasma clade by analyses of full-length or nearly full-length sequences of the 16S rRNA genes from more than 40 representative phytoplasmas (Namba *et al.*, 1993; Gundersen *et al.*, 1994; Seemüller *et al.*, 1994). The classification based on sequence analysis largely corresponds to the grouping obtained by RFLP analysis of PCR-amplified 16S rRNA genes. The few differences observed indicate that the sequences of large molecules, such as the 16S rRNA gene, reflect phylogenetic distance more accurately than restriction patterns which depend on significantly fewer genetic characters (Seemüller *et al.*, 1994).

Analysis of the spacer region between the 16S and 23S rRNA genes from more than 60 phytoplasma strains has resulted in a classification scheme similar to that derived from 16S rDNA data (Kirkpatrick *et al.*, 1994; Schneider *et al.*, 1995). More detailed differentiation of the closely-related phytoplasmas has been obtained from the sequence data of the 16/23S spacer regions. Nucleotide sequence analysis of the conserved ribosomal protein genes supported the designation of subclades and their phylogenetic positions within the phytoplasma clade as obtained by 16S rDNA sequence analysis (Gundersen *et al.*, 1994).

1.4 Molecular Biodiversity of Phytoplasmas

1.4.1 Phytoplasma Evolution

A hypothetical scheme for mycoplasma phylogeny has been constructed on the basis of normalization of rRNA phylogenetic tree branch lengths (Maniloff, 1996, 2000). Mycoplasmas originated with divergence of the acholeplasma-anaeroplasma-phytoplasma (AAP) branch from the *Streptococcus* phylogenetic branch about 600 million years ago. The ancestral mycoplasma was probably an acholeplasma (i.e., a facultative aerobe similar to streptococci) with obligate anaerobic anaeroplasmas arising later from the acholeplasma branch. The spiroplasma-entomoplasma-mycoplasma (SEM) branch diverged from the AAP branch about 470 million years ago, and split about 410 million years ago into a spiroplasma-entomoplasma branch and a mycoplasma branch. The phytoplasma branch arose on the AAP branch about 180 million years ago and, therefore, is phylogenetically distant from, and much younger than, the SEM branch plant and insect mycoplasmas. It was suggested that, in both major branches, genome reductions had occurred independently during their degenerative evolution. Thus, degenerative evolution of the AAP branch after the appearance of flowering plants has led to the phytoplasmas with 600 to 1,200 bp genomes, while evolution of the SEM branch produced the spiroplasma branch with 1,000 to 2,000 kb genomes and the entomoplasma, mesoplasma, mycoplasma, and ureaplasma branches with 600 to 1,200 kb genomes (Maniloff, 2000).

1.4.2 Molecular Markers

The following criteria for phylogenetically useful genes or gene products in prokaryotes have been proposed (Sogin *et al.*, 1972; Stackebrandt and Woese, 1981; Woese, 1987): 1) the gene must be universally distributed, since every organism must contain the gene in order for it to be a universal phylogenetic measure, 2) the gene product must have the same function in every organism, so that it has been under the same selective pressure in every organism, 3) the gene must not be subject to significant lateral transfer, which would obviate its use as a phylogenetic measure, 4) the gene base sequence must show clocklike behavior in terms of an accumulating random base change at a rate that allows changes over long genealogical times to be preserved, 5) the gene or gene product must be readily isolated and sequenced for it to be an experimentally feasible phylogenetic measure.

Pioneer studies have indicated that the small ribosomal subunit rRNAs meet these requirements (Fox *et al.*, 1980; Woese, 1987). The rRNA genes of the mollicutes are generally close to each other in the order 5' - 16S - 23S - 5S - 3', functioning as an operon (Fig. 1-1) (Sawada *et al.*, 1984). After comprehensive study of 5S and 16S rRNA sequences, it was demonstrated that the 16S rRNA sequences are the most useful phylogenetic tool because the longer 16S rRNA genes (about 1,540 bases) exhibit better clocklike behavior than do the smaller 5S rRNA genes (about 120 bases) and, quite serendipitously, 16S rRNA genes contain two phylogenetic clocks (Woese *et al.*, 1975). Some parts of the 16S rRNA sequence have evolved relatively slowly (i.e., have been conserved during microbial evolution), while other parts have evolved relatively rapidly. Hence, the slowly changing parts of the 16S rRNA sequence provide a phylogenetic measure of deep genealogical events, and the rapidly changing parts measure more recent genealogical events. Therefore, the 16S rRNA sequences have been extensively used for detection, identification, classification, and molecular evolution of phytoplasmas (Seemüller *et al.*, 1998).

However, the 16S rRNA gene does not always seem sufficiently variable to allow for the distinction of phytoplasmas that differ in host range or vector specificity. More variable genes should be used to differentiate among closely-related phytoplasmas. The first choice is the spacer region between 16S and 23S rRNA genes flanking a universal, highly-conserved tRNA^{Ile} gene that is less conserved than the 16S rRNA genes (Kirkpatrick *et al.*, 1994). The spacer region is shorter than the 16S rRNA gene (220-250 bp versus 1530 bp) and thus easier to sequence. Therefore, it can be used for finer differentiation of the closely-related strains (Kirkpatrick *et al.*, 1994; Smart *et al.*, 1996). The ribosomal protein genes are more variable in size and primary sequence than the highly-conserved 16S rRNA genes. Thus, ribosomal protein genes have a greater potential to reveal variations among closely-related strains (Gundersen *et al.*, 1996). The highly-conserved and ubiquitous elongation factor EF-Tu (*tuf*) gene has also proved to be an effective phylogenetic marker (Marcone *et al.*, 2000; Schneider *et al.*, 1997).

1.4.3 Classification of Phytoplasmas

Although mollicutes lack a cell wall, they were originally proposed by Neimark and London (1982) to be most closely related to gram-positive bacteria, and they originated from genome reductions from a walled ancestor. Woese (1987) and his coworkers (Woese *et al.*, 1980, 1985) postulated that the phylogeny of prokaryotes could be investigated through comparative sequence analyses of the highly-conserved 16S rRNA genes. They analyzed 16S rRNA oligonucleotide signature sequences of several *Mycoplasma*, *Spiroplasma*, and *Acholeplasma* species and found that these organisms constituted a rapidly evolving group that was the most closely related to several *Clostridium* species (Woese *et al*, 1985). However, a more comprehensive analysis of mollicutes phylogeny was conducted by Weisburg *et al.* (1989) who cloned and sequenced nearly full-length 16S rRNA genes from approximately 50 mollicutes species. They found that all of the mollicutes and their walled relatives formed a distinct clade within the low G+C gram-positive bacteria. At least five distinct groups or subclades could be organized among the 50 mollicutes species (Weisburg *et al.*, 1989). This approach has been used to determine genetic relationships of culturable mollicutes with each other and with walled bacteria (Weisburg *et al.*, 1989; Maniloff, 1992; Razin *et al.*, 1998). The current classification of mollicutes and the properties distinguishing the established taxa are presented in Table 1-1.

Since the first discovery of phytoplasmas (Doi *et al.*, 1967), when they were termed MLOs because of their resemblance in morphology and ultrastructure to animal mycoplasmas, members of the class *Mollicutes*, the taxonomic status of phytoplasmas has been uncertain due to the inability to culture them *in vitro*. Lim and Sears (1989, 1992) and Kuske and Kirkpatrick (1992) conducted the first phylogenetic studies of phytoplasmas by cloning and sequencing the complete 16S rRNA genes of phytoplasmas. They revealed that Michigan aster yellows (O-AY) and California aster yellows (SAY), and WX phytoplasmas are more closely related to certain culturable mollicutes such as acholeplasmas than to other bacteria.

Sequence analyses showed that the 16S rRNA gene of the O-AY phytoplasma was 99.5% homologous to SAY phytoplasma but only 89% homologous to WX phytoplasma, indicating a significant divergence between AY and WX phytoplasmas in sequence similarity. This conclusion also was supported by biological properties such as symptomatology and vector specificity, DNA-DNA hybridization, as well as serological and DNA hybridization analyses (Lin and Chen, 1985; Kirkpatrick *et al.*, 1987; Lee *et al.*, 1992b; Sears *et al.*, 1989; Kuske *et al.*, 1991a, b). Phylogenetic analyses of these three phytoplasmas clearly showed that they were more closely related to *Acholeplasma* *laidlawii* than to any true *Mycoplasma* species. In addition, these three phytoplasmas were more closely related to each other than they were to any other culturable mollicutes. This conclusion has also been supported by their small genome size (between 600 and 1,150 kb) (Lim and Sears, 1991; Neimark and Kirkpatrick, 1993) and low G+C content (23.0 to 26.2 mol %) (Kollar and Seemüller, 1989), by the finding of only two rRNA operons in the phytoplasmas (Schneider and Seemüller, 1994), and by the presence of an $rRNA^{Ile}$ gene in the spacer region between the 16S and 23S rRNA genes (Kirkpatrick *et al.*, 1994). In addition, sequence analyses of the ribosomal protein genes revealed that phytoplasmas, like acholeplasmas, used UGG as a tryptophan codon, whereas mycoplasmas, spiroplasmas and ureaplasmas used the stop codon UGA to code for tryptophan (Lim and Sears, 1992). The comprehensive phylogenetic analyses of the 16S rRNA gene, 16/23S spacer region, and ribosomal protein gene sequences clearly indicated the placing of phytoplasmas as a distinct monophyletic clade within *Mollicutes* as presented in Figure 1-2 (Namba, *et al.*, 1993; Gundersen *et al.*, 1994; Lim and Sears, 1992; Seemüller *et al.*, 1994; Toth *et al.*, 1994). This resulted in replacing the trivial name MLO with the term phytoplasma (International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes*, 1993).

Because of the inability to culture phytoplasmas *in vitro*, the criteria of modern bacterial taxonomy, in which genotypic, phylogenetic, and phenotypic information is integrated (Stackebrandt and Goebel, 1994), cannot be employed in phytoplasmology. In the absence of the phenotypic markers used to classify mollicutes, taxonomic affiliations presently cannot be resolved in the conventional way. Thus, a provisional classification of such unculturable bacteria, which are extensively characterized by molecular techniques, has been proposed using a *Candidatus* prefix (Murray and Schleifer, 1994). The Working Team on Phytoplasmas of the International Research Program of Comparative Mycoplasmology (IRPCM) of the International Organization for Mycoplasmology (IOM) has proposed adoption of this new classification based on 16S rRNA gene sequences and description of each subclade (major group) defined by the tentative species level as a *Candidatus* Phytoplasma species. The International Committee on Systematic Bacteriology (ICSB) Subcommittee on the Taxonomy of *Mollicutes* (1993, 1997) has agreed to this proposal and adopted the policy of basing phytoplasma taxonomy on phylogeny. So far, five *Candidatus* species names for phytoplasmas have been published: *Candidatus* phytopasma aurantifolia (Zreik *et al*., 1995), *Canadidatus* P. australiense (Davis *et al*., 1997), *Canadidatus* P. australasia (White *et al*., 1998), *Canadidatus* P. japonicum (Sawayanagi *et al*., 1999), and *Canadidatus* P. fraxini (Griffiths *et al*., 1999).

Since the introduction of PCR to amplify nearly full-length 16S rRNA genes from phytoplasmas (Deng and Hiruki, 1990b, 1991a), RFLP analysis of PCR-amplified 16S rRNA genes has been widely employed for the classification of phytoplasmas. Ahrens and Seemüller (1992) were the first to use RFLP analysis of PCR-amplified phytoplasma 16S rRNA genes to differentiate 17 phytoplasma isolates into four distinct groups. A similar RFLP analysis established approximately 12 major groups and several subgroups (Lee *et al*., 1993; Schneider *et al*., 1993). A total of 14 major groups and 38 subgroups have been identified on the basis of comprehensive RFLP analysis of PCR-amplified 16S rRNA genes (Lee *et al*., 1993, 1998b; Gundersen *et al*., 1994). The finer differentiation of the closely-related phytoplasmas has been done by the analyses of 16S ribosomal protein genes and the 16/23S spacer region (Gundersen *et al*., 1996; Schneider *et al*., 1995; Smart *et al*., 1996).

Comprehensive classification of phytoplasmas has been achieved by the examination of the full-length or nearly full-length phytoplasma 16S rDNA sequences (Namba *et al*., 1993; Seemüller *et al*., 1994; Kirkpatrick *et al*., 1992; Gunderson *et al*., 1994). Analysis of 57 phytoplasmas revealed 20 major groups and their relationships to other mollicutes (Seemüller *et al*., 1998), as presented in Figure 1-3. Eight groups were added to the new classification on the basis of branching patterns and by differences in the 16S rDNA sequence homology of at least 2.3% or greater, while the IRPCM Working Team on Phytoplasmas agreed to a threshold of 2.5% to separate the stolbur phytoplasma group from the AY agents (ICSB Subcommittee on the Taxonomy of *Mollicutes*. 1997). A total of 246 phytoplasmas were assigned to 20 groups on the basis of DNA-based and/or serological methods.

The number of phytoplasma strains varies greatly with each group or subgroup. The largest group is the AY group which is divided into 11 subgroups (I-A through I-K), and includes 103 phytoplasmas according to a scheme proposed by Lee *et al*. (1993, 1998b) and Gundersen *et al.* (1994, 1996), on the basis of 16S rDNA RFLP or putative restriction site analysis. Recently, on the basis of RFLP analysis of an 1,800 bp fragment comprising the entire 16S rRNA and the 16S/23S spacer region which was more suitable for AY-group phytoplasma differentiation than a 1240 bp fragment of the 16S rRNA gene, three new subgroups were added for a total of 14 subgroups (Marcone *et al.*, 2000). Among the 14 subgroups in the AY group, subgroup I-B is the largest one and is associated with diseases of more than 60 plant species (Seemüller, *et al.*, 1998; Lee *et al.*, 1998b). However, the subgroups I-D, I-E, I-F, and I-H include only one phytoplasma each. The 143 non-AY phytoplasmas were assigned into 19 major groups, including 58 taxonomic units, on the basis of DNA-based or serological studies. Certain major groups, such as the BWB (buckthorn witches'-broom) group and the CirP (cirsium phyllody) group, contain only one taxon whereas other groups, such as the X-disease group, contain up to 11 taxa.

Sequence analyses of more than 60 phytoplasma isolates showed that phylogenetic trees constructed from 16/23S spacer regions were in complete agreement with the major phytoplasma clades established by full-length 16S rRNA sequences (Kirkpatrick *et al.*, 1994; Schneider *et al.*, 1995; Smart *et al.*, 1996). The finer level of subgroup differentiation within each major group has been revealed by sequence analyses of both 16/23S spacer regions and ribosomal protein genes (Gundersen *et al.*, 1996; Schneider *et al.*, 1995; Jomantiene *et al.*, 1998). Although the *tuf* gene was considered to be more variable than the 16S rRNA gene, the subgroups 16SrI-D, -L and –M could not be differentiated by analyses of the *tuf* gene (Marcone *et al.*, 2000; Schneider *et al.*, 1997).

1.4.4 Geographic Distribution of Phytoplasmas

Phytoplasmas occur worldwide, but there are significant differences in the distribution of members belonging to various groups and subgroups. For example, subgroups I-A and I-D phytoplasmas have been reported only in North America and Asia, respectively, whereas members of subgroup I-B are widespread worldwide. Phytoplasmas of the stolbur and ash yellows groups have been identified only in Europe and North America, respectively, whereas phytoplasmas of the FBP group are only known in Africa, Asia and Australia. Most AP group phytoplasmas have been found in Europe, while most CP group phytoplasmas have been identified in North America. However, the current information about distribution is likely to be just a temporary picture and will possibly change with further research (Seemüller *et al.*, 1998).

1.4.5 Host Specificity of Phytoplasmas

The natural hosts of phytoplasmas are plants and insects. Host specificity varies greatly among phytoplasma strains. For example, members of subgroup I-B in the AY group have a wide host range worldwide (Lee *et al.*, 1993, 1998b; Gundersen *et al.*, 1994, 1996; Seemüller *et al.*, 1998). Sunn hemp witches'-broom and stolbur phytoplasmas have also been identified in many plant species. However, some phytoplasmas have very narrow host ranges in plants. Examples are paulownia witches'-broom (Hiruki, 1999), blueberry stunt (Schneider *et al.*, 1993), and maize bushy stunt (Nault *et al.*, 1979) phytoplasmas which have been found in only one plant species each.

The plant host range of a given phytoplasma in nature is determined largely by the susceptibility of plants and by the host affinity of natural insect vector species that are capable of transmitting the phytoplasma. Phytoplasmas also differ considerably in insect vector specificity. Some phytoplasmas have low insect vector specificity, whereas others have very high vector specificity. Examples of low insect vector specificity are SAY phytoplasma which is transmitted by 24 species of leafhoppers; peach-X disease phytoplasma which is transmitted by at least 15 species of leafhoppers; and CPh phytoplasma which is transmitted by at least nine species of leafhoppers (Mäurer *et al.*, 1993; Toth *et al.*, 1994). Some phytoplasmas with high insect vector specificity are beet leafhopper-transmitted virescence phytoplasma, American EY phytoplasma, SCWL phytoplasma, lofah witches'-broom phytoplasma, and PD phytoplasma. These phytoplasmas appear to be transmitted by only one or a few vector species (Vega *et al.*, 1993).

However, host specificity is still not well understood and the differentiation of phytoplasmas at the host specificity level is difficult. It seems not always possible to distinguish phytoplasmas differing in host specificity by RFLP analysis of PCR-amplified conserved sequences (Seemüller *et al.*, 1998). Therefore, other molecular markers, such as transmission genes and pathogenesis genes, or other research methods, such as HMA with its high sensitivity to detect mutations, should be used in studies examining genetic diversity.

1.4.6 Vector-Phytoplasma-Plant Relationships

The recent development of molecular techniques has made it possible to identify the phytoplasma associated with a given disease or insect vector for the study of vector-phytoplasma-plant relationships. Previous studies have revealed that the identities of phytoplasmas are not parallel to the symptoms they induce on susceptible plant species. Similar symptoms can be induced by various distinct types of phytoplasmas (Bertaccini *et al.*, 1995; Bianco *et al.*, 1993; Daire *et al.*, 1993; Gibb, *et al.*, 1995; Padovan *et al.*, 1995; Prince *et al.*, 1993; Wang and Hiruki, 2001a, b), whereas several disparate symptom types can be induced by the closely-related phytoplasmas (Jomantiene *et al.*, 1998; Lee *et al.*, 1992;Vibio *et al.*, 1996). For example, both canola yellows and dandelion yellows are associated with at least two distinct phytoplasmas in subgroups I-A and I-B, respectively (Wang and Hiruki, 2001a). Tomato big-bud diseases are associated with four different phytoplasmas from the AY, FBP, EY, and WX groups in different geographical origins (Boudon-Padieu *et al.*, 1996; Gibb, *et al.*, 1996; Lee *et al.*, 1993; Schneider *et al.*, 1993).

A given disease develops as the result of intricate vector-phytoplasma-plant interactions. Certain insect vectors are capable of transmitting more than one type of phytoplasma. For example, *Colladonus montanus* and *Scaphytopious acutus delongi* are common vectors that transmit phytoplasmas associated with both WX and SAY (Nielson, 1979; Tsai, 1979). *Aphrodes bicinctus* is the major vector that transmits North American aster yellows, clover phyllogy, strawberry green petal, and stolbur disease with less efficiency (Brcák, 1979; Tsai, 1979). *Macrosteles laevis* transmits European aster yellows (subgroup I-B), stolbur, clover phyllody, clover dwarf, primula yellows (subgroup I-B) and onion yellows (Brcák, 1979; Nielson, 1979), but cannot transmit North American (subgroup I-A) and California (subgroup I-B) AY strains (Brcák, 1979).

However, *M. fascifrons*, a species similar to *M. laevis*, can transmit both American AY strains and the European AY. This may explain why the North American AY strain (subgroup I-A) has not been found in Europe, except for one rare example associated with gladiolus virescence (Vibio, *et al.*, 1996).

The number of phytoplasmas associated with a given plant species depends on not only plant susceptibility to phytoplasma infection, but also on the geographic distributions of various vectors as well as the preferential host(s) of each vector. Although many plant species appear to be infected only by a specific phytoplasma in nature, there are plant species that are infected by several distinct phytoplasmas. For example, peach can be affected by WX in North America (Kirkpatrick *et al.*, 1987), PD in China, peach leaf chlorotic roll in the United States and Europe (Gundersen *et al.*, 1996), and peach rosette (Marcone *et al.*, 1995) in Europe. Clover can be affected by CPh (Lee *et al.*, 1992), CP (Chen and Hiruki, 1975), CYE (Sinha and Benhamou, 1983), and clover dwarf (Brcák, 1979). In most cases, each distinct phytoplasma is transmitted by a unique vector in different geographic regions (Brcák, 1979; Nielson, 1979).

Since different phytoplasmas can infect the same plants and vectors in nature, the occurrence of mixed infections recently have been revealed by nested PCR assays. Most mixed infections are associated with a predominant phytoplasma and one to several other phytoplasmas present in lower titers (Alma *et al.*, 1996; Bianco *et al.*, 1993; Lee *et al.*, 1994, 1995; Marcone *et al.*, 1996). In Europe, host plants and vectors of AY, STOL, and EY group phytoplasmas overlap, and these pathogens are commonly found in mixed infections (Lee *et al.*, 1998b). However, little is known about the interactions among the phytoplasmas in a single infected plant.

1.5 The objectives of this study

On the basis of the above analyses of the problems existing in genetic diversity studies of phytoplasmas, this research has focused on the development of highly specific and rapid diagnostic procedures, based on molecular biological approaches that could lead to an internationally acceptable taxonomy of phytoplasmas. The following objectives will be addressed:

1. To investigate the sensitivity of HMA in detecting DNA mutations of phytoplasmas by artificially introducing substitutions and insertions/deletions.
2. To test the suitability of HMA for classifying phytoplasmas by using representative phytoplasmas in different major groups.
3. To select a suitable gene for using HMA for the classification of phytoplasmas.
4. To investigate the genetic diversity of phytoplasmas in the CP group.
5. To identify field samples collected from Canada, Europe, and Asia and to determine their genetic relationships by HMA.
6. To study the molecular evolution of phytoplasmas by analyzing the 16S rRNA gene and 16/23S spacer region sequences.

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Table 1-1. Major characteristics and taxonomy of the class *Mollicutes*[a]

Classification	Current no. of recognized species	Genome size (kb)	Mol% G+C of genome	Cholesterol requirement	Distinctive properties	Habitat
Order I: Mycoplasmas						
Family I: Mycoplasmataceae						
Genus I: Mycoplasma	102	580-1,350	23-40	Yes	Optimum growth at 37°C	Humans, animals
Genus II: Ureaplasma	6	760-1,170	27-30	Yes	Urea hydrolysis	Humans, animals
Order II: Entomoplasmatales						
Family I: Entomoplasmataceae						
Genus I:Entomoplasma	5	790-1,140	27-29	Yes	Optimum growth at 30°C	Insects, plants
Genus II: Mesoplasma	12	870-1,100	27-30	No	Optimum growth at 30°C 0.04% Tween 80 required in serum-free medium	Insects, plants
Family II: Spiroplasmataceae						
Genus I: Spiroplasma	33	780-2,220	24-31	Yes	Helical motile filaments; Optimum growth at 30-37°C	Insects, plants
Order III Acholeplasmatales						
Family I: Acholeplasmataceae						
Genus : Acholeplasma	13	1,500-1,650	26-36	No	Optimum growth at 30-37°C	Animals, some plants, insects
Order IV: Anaeroplasmatales						
Family: *Anaeroplasmataceae*						
Genus I: Anaeroplasma	4	1,500-1600	29-34	Yes	Oxygen-sensitive anaerobes	Bovine/ovine rumen
Genus II: Asteroleplasma	1	1,500	40	No	Oxygen-sensitive anaerobes	Bovine/ovine rumen
Undefined taxonomic status						
Phytoplasma	ND[b]	530-1,350	23-29	Not known	Uncultured in vitro	Insects, plants

[a] Modified from Razin *et al.*, 1998.

[b] The taxonomic status of the uncultured phytoplasmas is not defined (ND) as yet; five Candidatus Phytoplasma species have already been published.

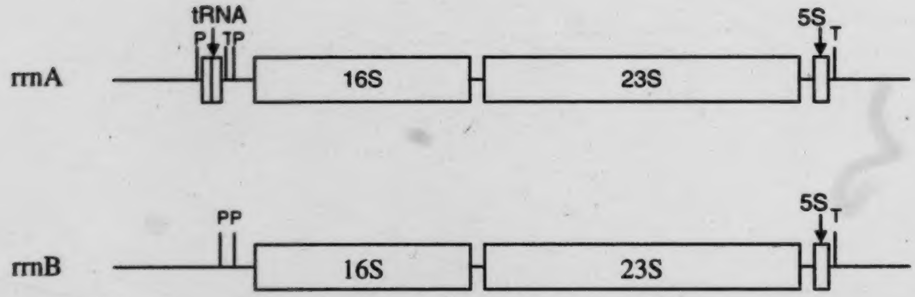


Figure 1-1. General organization of ribosomal RNA genes of mollicutes (Sawada *et al.*, 1984). P and T represent putative promoter and terminator respectively. Generally, two rRNA operons, rrnA and rrnB, are presented in mollicutes.

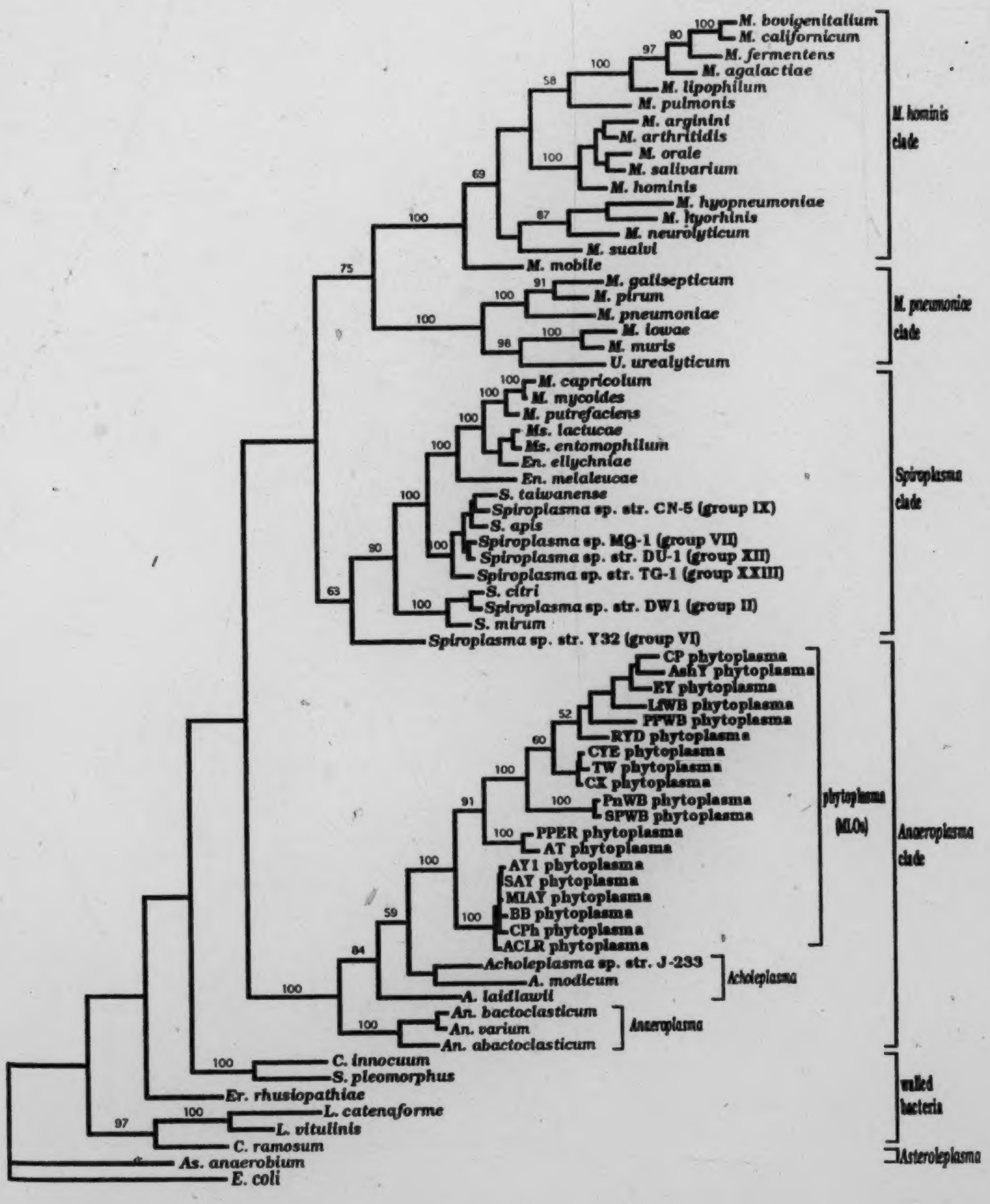
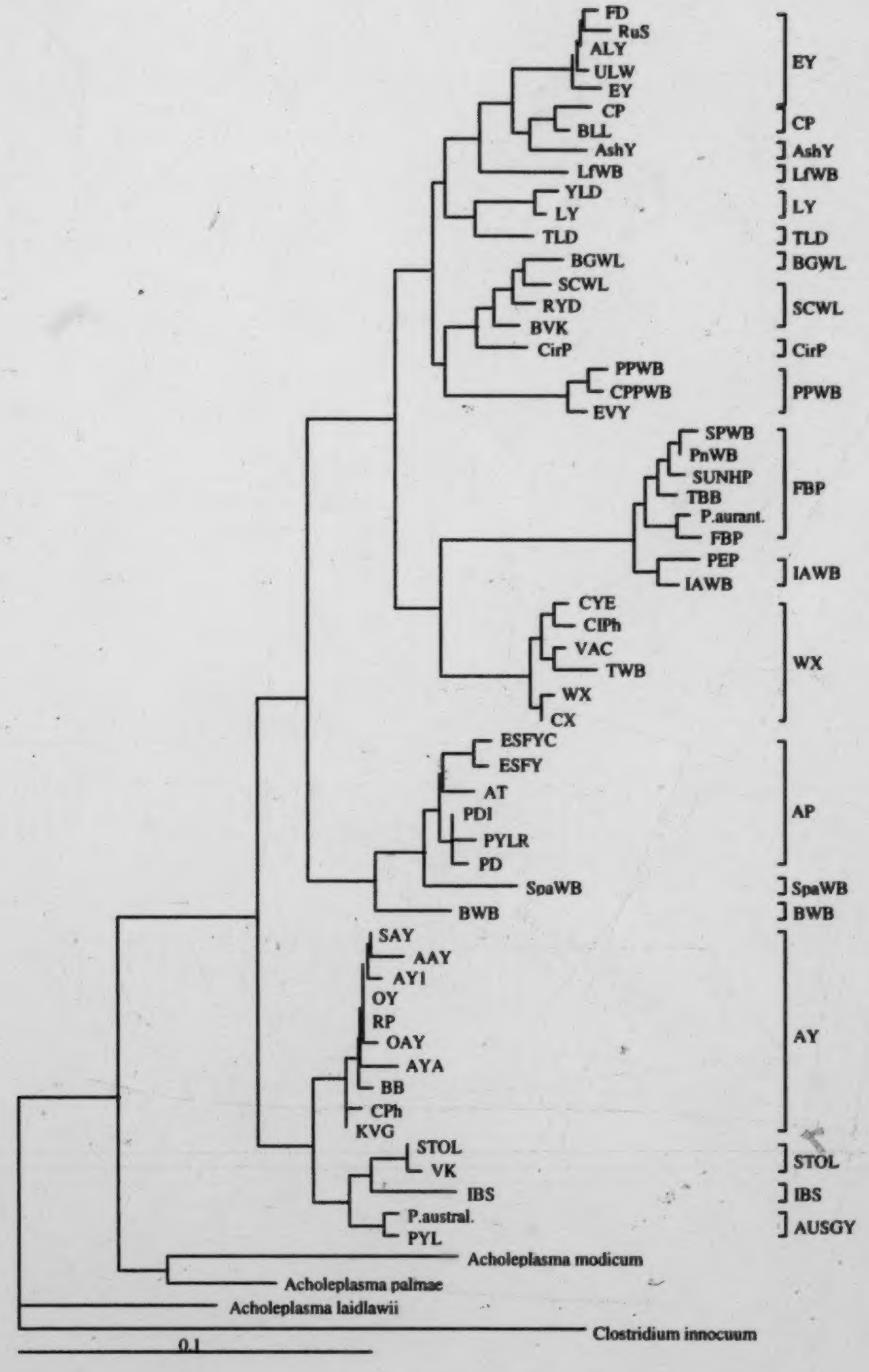


Figure 1-2. The relationship among members of the class Mollicutes and several representative walled bacteria (Gundersen *et al.*, 1994).

Fig 1-3. A revised phylogenetic dendrogram of the phytoplasmas based on the analysis of 16S rRNA gene sequences (Seemüller *et al.*, 1998). The bar represents a phylogenetic distance of 10%. Phylogenetic groups defined are shown in the right hand column. Phytoplasma strain abbreviations are: PYL, phormium yellow leaf; P. Austral., *Candidatus* Phytoplasma australiense (AUSGY); IBS, Italian bindweed stolbur; VK, Vergilbungskrankheit (grapevine yellows); STOL, stolbur of pepper; KVG, clover phyllody/Germany; CPh, clover phyllody/Canada; BB, tomato big bud/Arkansas; AYA (formerly ACLR), aster yellows from apricot; OAY, Oenothera aster yellows; SAY, western severe aster yellows; BWB, buckthorn witches'-broom; SpaWB (formerly SPAR), spartium witches'-broom; PD, pear decline/Germany; PYLR, peach yellow leaf roll; PDI, pear decline/Italy; AT, apple proliferation; ESFY, European stone fruit yellows/Germany; ESFYC, European stone fruit yellows/Czech Republic; CX, Canadian X-disease; WX, western X-disease; TWB, tsuwabuki witches'-broom; VAC, vaccinium witches'-broom; ICPh, Italian clover phyllody; CYE, clover yellow edge; IAWB, Italian alfalfa witches'-broom; PEP, picris echioides phyllody; FBP, faba bean phyllody; P. aurant., *Candidatus* P. Aurantifolia (WBDL); TBB, tomato big bud/Australia; SUNHP, sunn hemp witches'-broom; PnWB, peanut witches'-broom; SPWB, sweet potato witches'-broom; EVY, echium vulgare yellows; CPPWB, Caribbean pigeon pea witches'-broom; PPWB, pigeon pea witches'-broom; CirP, cirsium phyllody; BVK, from leafhopper *Psammotettix cephalotes*; RYD, rice yellow dwarf; SCWL, sugarcane white leaf; BGWL, Bermuda grass white leaf; TLD, Tanzanian lethal decline of coconut; LY, coconut lethal yellowing little leaf; CP, clover proliferation; EY, elm yellows; ULW, elm yellows/France; ALY, alder yellows; RrS, rubus stunt; FD, flavescence dorée.


FD
RuS
ALY
ULW
EY
CP
BLL
AshY
LfWB
YLD
LY
TLD
BGWL
SCWL
RYD
BVK
CirP
PPWB
CPPWB
EVY
SPWB
PnWB
SUNHP
TBB
P.aurant.
FBP
PEP
IAWB
CYE
ClPh
VAC
TWB
WX
CX
ESFYC
ESFY
AT
PDI
PYLR
PD
SpaWB
BWB
SAY
AAY
AY1
OY
RP
OAY
AYA
BB
CPh
KVG
STOL
VK
IBS
P.austral.
PYL
Acholeplasma modicum
Acholeplasma palmae
Acholeplasma laidlawii
Clostridium innocuum
0.1
EY
CP
AshY
LfWB
LY
TLD
BGWL
SCWL
CirP
PPWB
FBP
IAWB
WX
AP
SpaWB
BWB
AY
STOL
IBS
AUSGY

II

Sensitivities of Heteroduplex Mobility Assay (HMA) in Detecting DNA mutations for Differentiation of Closely Related Phytoplasma Isolates*

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* A version of this chapter has been published. Wang, K., and Hiruki, C. 2000. J. Microbiol. Methods 41:59-68.

2.1 Introduction

Phytoplasmas are wall-less prokaryotes associated with the diseases of several hundred plant species (McCoy *et al.*, 1989). Due to the inability thus far to isolate phytoplasmas in pure culture, phytoplasmas were once differentiated on the basis of the symptoms they induced, the host plant affected, and the specific insect vector in classical plant mollicutes studies. However, the recent development of phytoplasma-specific molecular probes for serological and DNA hybridization analysis has significantly improved phytoplasma identification and classification (Kirkpatrick 1989). The intensive use of restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified conserved genes has contributed to our understanding of the phylogeny and taxonomy of phytoplasmas (Deng and Hiruki, 1990, 1991a, 1991b; Schneider *et al.*, 1993; Lee *et al.*, 1993). Subsequently, the phylogenetic tree of phytoplasmas has been established on the basis of RFLP and sequence analyses of the 16S rRNA genes, the 16/23S spacer regions, and the 16S ribosomal protein genes (Namba *et al.*, 1993; Gundersen *et al.*, 1994, 1996; Seemüller *et al.*, 1994, 1998; Smart *et al.*, 1996). However, RFLP analysis is based on the presence or absence of a restriction enzyme cleavage site within a DNA fragment. There are two major problems associated with the use of RFLP: first, its inability to detect a large fraction of mutations and polymorphisms (Myers *et al.*, 1987) since the chances that a mutation will alter a restriction site are low. Secondly, it may incorrectly increase the phylogenetic distance between closely-related phytoplasma isolates (Seemüller *et al.*, 1994; Kuske and Kirkpatrick, 1992). DNA sequence analyses of 16S rDNA of more than 20 phytoplasma isolates in previous studies have indicated that the RFLP-based classification of phytoplasmas in certain groups does not fully coincide with the phylogenetic relationships of the organisms (Seemüller *et al.*, 1994; Kuske and Kirkpatrick, 1992). Therefore, different RFLP patterns do not always indicate significant phylogenetic distances. To determine whether a phytoplasma isolate belongs to a new subgroup, 16S rDNA sequence data must be analyzed (Sawayanagi *et al.*, 1999).

Denaturing gradient gel electrophoresis (DGGE) has been used to estimate the levels of sequence diversity in given phytoplasma isolates (Ceranic-Zagorac and Hiruki, 1996). While changes in a single base are distinguishable by DGGE analysis (Wartell *et al.*, 1998), this technique is considerably complicated and laborious, and may not provide an easily derived estimate of the extent of genetic divergence between sequences of different DNAs. Therefore, new strategies for reliable identification and classification of phytoplasma isolates would be valuable.

Heteroduplex mobility assay (HMA) has been used for the study of the variability of human immunodeficiency virus type 1 (HIV_1) surface envelope (*env*) glycoprotein coding sequences (Delwart *et al.*, 1993, 1994). Genetic relationships between multiple viral DNA template molecules can be rapidly evaluated by this method. The sensitivity of HMA for detecting DNA mutation is higher than any other techniques currently available, except for exhaustive DNA sequencing studies (Delwart *et al.*, 1993, 1994). HMA has recently been applied to establish the molecular relatedness of phytoplasma isolates (Zhong and Hiruki, 1994; Ceranic-Zagorac and Hiruki, 1996; Cousin *et al.*, 1998).

The objectives of this chapter were to determine the sensitivity of HMA in detecting the mutations of 16S rDNA fragments derived from the aster yellows phytoplasma isolate 27 (AY27), and to detect the mixed DNA fragments by HMA.

2.2 Materials and Methods

2.2.1 Preparation of the partial 16S rDNA fragment

A 16S rDNA fragment (1.2 kb) was amplified from AY27 as previously described elsewhere (Wang *et al.*, 1998), and subjected to digestion by restriction enzyme *Alu*I (GIBCO BRL, Ontario, Canada) following the manufacturer's instructions. The digestion products were analyzed by electrophoresis in a 1% low melting point agarose (GIBCO) gel in 1× TAE buffer and stained in ethidium bromide. The DNA fragment of about 352 bp in length was cut and purified by the Agarose Gel DNA Extraction Kit (Boehringer Mannheim, Mannheim, Germany). The DNA fragment was eluted in 25 µl of TE buffer (10 mM Tris·HCl, 0.1 mM EDTA pH 8.0) and stored at –20°C.

2.2.2 Cloning of the partial 16S rDNA

The purified partial 16S rDNA fragment (352 bp) was ligated in PCR-Script Amp SK(+) vector (Stratagene, Lo Jolla, CA, USA) under the following conditions: 10 ng of vector, 1× ligation buffer, 1 mM rATP, 80 ng of DNA, 10 U of restriction enzyme *SrfI*, and 3 U of T4 DNA ligase in 10 µl of reaction mixture. The ligation reaction was incubated at room temperature for 1 h followed by heating at 65°C for 10 min to inactivate the ligase. Then 2 µl of ligation reaction was used for transformation in 40 µl of *Escherichia coli* competent cells in a 14 ml Falcon tube by heat pulse at 42°C for 30 sec in a water bath. The tube was immediately placed on ice for 2 min and 450 µl of SOC liquid medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 12.5 mM $MgCl_2$, 12.5 mM $MgSO_2$, 20 mM glucose) was added. The competent cells were recovered by incubation at 37°C with shaking at 250 r.p.m. for 1 h. The 100 µl of transformation mixture was spread on an LB-Ampicillin (50 µg/ml) agar plate with IPTG (isopropyl-1-thio-β-D-galactopyranoside) and X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) and incubated at 37°C for 16 h.

2.2.3 Selection of clones and confirmation by PCR

The white colonies were picked up using toothpicks and cultured in 200 µl of LB liquid medium with 10 µg of Ampicillin in a 96-well culture plate at 37°C for 16 h. Meanwhile, the colonies were screened by PCR using primers T3 (5' AATTAACCCTCACTAAAGGG 3') and T7 (5' GTAATACGACTCACTATAGGGC 3'). The PCR conditions were as follows: denaturation at 94°C for 40 sec, annealing at 45°C for 45 sec, and extension at 72°C for 50 sec, 40 cycles. Before the cycling, DNA was denatured at 94°C for 3 min and was incubated for 7 min after cycling. Each 5 µl of PCR products was used for electrophoresis in a 1% agarose gel.

2.2.4 Preparation of insertion-vector

Clones containing the partial 16S rDNA insert were selected for extraction of insertion-plasmids. The cells were collected from 50 ml of cell cultures by centrifugation at 10,000 *g* for 10 min at 4°C, and then resuspended in 4 ml of lysozyme solution (250 mM Tris-HCl, pH 8.0; 10mM EDTA, pH 8.0; 0.9 g/ml glucose; 5 mg/ml lysozyme), incubated on ice for 5 min. Eight microliters of a freshly-prepared NaOH-SDS solution (0.2 M NaOH, 1% SDS) were added to the lysozyme solution and incubated on ice for 5 min. The bacterial genomic DNA was precipitated by adding 6 ml ice-cold potassium acetate solution and spun at 15,000 *g* for 15 min. The supercoiled plasmid DNA in the supernatant was precipitated by adding 0.6 volume of isopropanol and pelleted by centrifugation at 15,000 *g* for 30 min. The final pellet was rinsed with 70% ethanol and dissolved in 400 µl TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0). The plasmid DNA was treated by DNase free RNase at a rate of 20 µg/ml and extracted by phenol once and phenol : chloroform : isoamyl alcohol (25:24:1) once. The final plasmid DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volume of ethanol, and dissolved in 100 µl of TE buffer. For the preparation of the insertion-plasmid vector, the plasmid was digested by *Hpa*I (GIBCO/BRL, Gaithersburg, MD) following the instructions provided by the manufacturer and extracted with phenol : chloroform (1:1), precipitated, dried, and dissolved in ddH_2O.

2.2.5 Preparation of adapters

A series of oligonucleotides were designed as in Table 2-1. Equal amounts of the complementary oligonucleotides were mixed together with presence of 20 mM Tris-HCl (pH 8.0) and 50 mM NaCl annealing buffer. After denaturation at 94°C for 1 min, the oligonucleotides were annealed at room temperature. The adapters were used for ligation reactions without further purification.

2.2.6 Mutations of 16S rDNA

The adapters were inserted into the partial 16S rDNA insertion-vector as described above. Briefly, 1 µl of 1 µM adapter was ligated into 1 µl of insertion-vector (10 ng/µl). The ligated insertion-vector (2 µl) was transformed into *E. coli* cells by heat pulse. The transformed cells were spread on LB agar plates containing 50 µg Ampicillin per milliliter of medium and incubated at 37°C overnight.

2.2.7 Confirmation of mutations by DNA sequencing

Clones containing the mutated partial 16S rDNA fragment derived from AY27 were prescreened by PCR. Each clone was amplified by primers T3 and T7. The PCR products were subjected to electrophoresis in a 5% polyacrylamide gel. The selected clones were further analyzed by DNA sequencing using the Dye Terminator Cycle Sequencing Core

Kit (Perkin Elmer Cetus, Norwalk, CT, USA) to confirm the insertion and orientation of the adapters.

2.2.8 HMA sensitivity

The clones containing adapters with the same orientation were selected and used for PCR amplification by primers T3 and T7. Each 4.5 µl of PCR products representing adapters from L1 to L9 was mixed with 4.5 µl of PCR products containing adapter L1 and 1 µl of 10× annealing buffer (100 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 1 M NaCl) was added. After denaturing at 98°C for 4 min, the DNA fragments were hybridized at 37°C overnight. The products of the hybridization reactions were subjected to electrophoresis in a 5% polyacrylamide gel at 250V for 3 h at room temperature in Protean II (Bio-Rad) with a cooling system. The DNA bands were stained in ethidium bromide and visualized under a UV transilluminator.

2.2.9 Detection of two DNA fragment mixture by HMA

The mutants L1-10 and L4-6 with a 3-base substitution and a clone L5-9 without 10-base adapter insertion were used for PCR amplification by primers T3 and T7 respectively. The PCR products amplified from mutant L4-6 and clone L5-9 were artificially mixed in equal volume. Then 4.5 µl of the DNA mixture and 4.5 µl of PCR product from L1-10 were mixed in equal volume with 1× annealing buffer. To identify the heteroduplex bands, the PCR products were also mixed in pairs. The following procedures were the same as those for the test of HMA sensitivity.

2.3 Results

2.3.1 Clones of the partial 16S rDNA

The clones containing the partial AY27 16S rDNA fragment were obtained by heat pulse transformation. Sixteen white colonies and two blue colonies were selected and screened by PCR. The results showed that the selected white clones contained the target DNA fragment (data not shown). One clone showing 16S rDNA insertion was selected for DNA sequencing. The results confirmed that the DNA inserted in the clone was 16S rDNA fragment of AY27.

2.3.2 Mutation of the partial 16S rDNA

Twenty-four colonies from each mutation were selected to screen for the insertion of adaptors by PCR. On average, six clones out of 24 randomly selected clones contained adaptors. Since two different orientations were possible for each adapter inserted into a vector, five clones with insertion for each adapter were sequenced. Twenty-five clones with both orientations of each adapter were obtained and used for the test of HMA sensitivity.

2.3.3 HMA sensitivity in detecting DNA mutations

In detecting DNA mutations, heteroduplexes were formed by mixing DNA fragments amplified from a series of mutants. When different pairs of amplified PCR products were denatured and reannealed, a single band was observed in agarose gels (data not shown). However, the same DNA mixtures resulted in two additional heteroduplex bands in addition to the comigrating homoduplex bands in a polyacrylamide gel (Fig. 2-1), which indicated that the mismatched or unpaired sequences affects their mobilities in a polyacrylamide gel.

Compared to mutant L1, mutants L2, L3, L4, and L5 had 1-, 2-, 3-, 4- base substitutions at a locus respectively, whereas L6 had two single-base substitutions. When PCR products amplified from mutants L2 to L5 were mixed with mutant L1 sequence, the heteroduplexes with only mismatched nucleotides were formed. Subsequently, HMA detected a single 2-base substitution out of 529 bp DNA fragment (Fig. 2-1, lane 5), and the heteroduplex mobility distances were generally proportional to the degree of mismatch. HMA differentiated the single-base substitutions between mutants L2 and L3, L3 and L4, L4 and L5, although it failed to detect a single-base substitution (L2) (Fig. 2-1, lane 3) and two single-base substitutions (L6) (Fig. 2-1, lane 4).

The mutants L7, L8, and L9 contained 1-, 2-, and 3- base deletions in comparison with mutant L1. After mixing the PCR product amplified from mutant L1 with those from mutants L7, L8, and L9 (Fig. 2-1, lanes 8, 9, and 10, respectively), heteroduplex bands were observed in all combinations, indicating that a single-base deletion was directly detected by HMA (Fig. 2-1, lane 8). The mobility distances increased with the number of unpaired bases in the gap. However, gaps caused greater retardation to heteroduplex mobilities than did mismatches.

2.3.4 Detection of two mixed DNA fragments

When the mixed DNA fragments amplified from L4-6 and L5-23 were combined with that from L1-10, only four heteroduplex DNA bands were observed (Fig. 2-2a, lines 4, 5, and 6). The DNA heteroduplexes formed between mutants L1-10 and L5-23 showed very similar mobilities to those formed between mutants L4-6 and L5-23 in a non-denatured polyacrylamide gel (Fig. 2-2a, lanes 2, and 3). Therefore, the lower two heteroduplex DNA bands corresponded to the heteroduplexes formed between L1-10 and L5-23, as well as heteroduplexes formed between L4-6 and L5-23. The top two heteroduplex DNA bands corresponded to the heteroduplexes formed between L1-10 and L4-6.

When mixed DNA fragments amplified from L4-6 and L5-9 were combined with that amplified from L1-10, six bands were observed (Fig. 2-2b, lanes 4, 5, and 6). Two bands corresponded to the heteroduplexes formed between L1-10 and L4-6, two to those between L1-10 and L5-9, and two to those between L4 and L5-9. The results agreed with the principle of HMA in which six heteroduplex DNA bands should be observed if three DNA fragments from different origins were mixed together. The results represented solid evidence that HMA is capable of detecting mixed DNA fragments and it is an ideal method to detect mixed infections by phytoplasmas.

2.4 Discussion

AY has been well-studied and the 16S rDNA has been sequenced completely (Seemüller *et al.*, 1994). After analyzing the sequence data, two *Alu*I restriction sites were found at positions 642 and 994 in the 16S rDNA sequence of AY. This 352 bp fragment contains an *Hpa*I restriction site and is suitable for DNA sequencing by a single sequencing reaction. In addition, the absence of an *Hpa*I site in the PCR-Script Amp SK(+) plasmid vector makes it possible to induce any mutations *in vitro* at the *Hpa*I site of 16S rDNA after the DNA fragment is inserted into the vector. Thus, the 16S rDNA fragment of AY is ideal material for producing a series of DNA point mutations to study the sensitivity of HMA.

Methods for rapid detection of single-base mutations are very important for the study of both genetic diseases and of the many problems in basic biology. In the study of human genetic diseases, several methods have been used to detect point mutations, including RNase cleavage (Myers *et al.*, 1985), chemical cleavage (Cotton *et al.*, 1988), denaturing gradient gel electrophoresis (Fischer and Lerman, 1980; Myers *et al.*, 1987; Sheffield *et al.*, 1989; 1992), temperature gradient gel electrophoresis (Lu *et al.*, 1995; Wartell *et al.*, 1998), single-stranded conformational polymorphism analysis (Orita *et al.*, 1989; Sarkar *et al.*, 1992), and heteroduplex analysis (Keen *et al.*, 1991; Ganguly *et al.*, 1993; Glavac and Dean, 1995). However, none of these techniques have been applied to detect single-base mutations in phytoplasma DNA. This study is the first reported case of HMA to detect single-base deletions/insertions or two-base substitutions in 16S rDNA fragments derived from AY27. Although HMA failed to detect the single-base substitutions, it was capable of differentiating a single-base difference between phytoplasmal DNA fragments when a suitable DNA fragment was used as a reference. In this study, while mutants L3 and L4, L4 and L5 had only a single-base difference, they had very different HMA profiles (Fig. 2-1, lanes 5, 6, and 7). Previous studies also indicated that the migration of heteroduplexes in non-denaturing polyacrylamide gels was different even when they contained the same number of nucleotide mismatches in different base compositions (White *et al.*, 1992; Ganguly *et al.*, 1993). In one reported case, the heteroduplex containing a C-T mismatch with the cytosine in the sense strand and the thymine in the antisense strand migrated more slowly than the homoduplex, whereas the heteroduplex containing the T-C mismatch with thymine in the sense strand comigrated with the homoduplex (Ganguly *et al.*, 1993). Adjacent mispaired or unpaired bases have also been shown to affect the migration of heteroduplexes (Wang and Griffith 1991; White *et al.*, 1992). Therefore, HMA provides an accurate technique to detect any mutations in phytoplasma functional genes and to study the molecular epidemiology of phytoplasmas.

The heteroduplexes containing gaps (Fig. 2-1, lanes 8, 9, and 10) showed marked electrophoretic retardation compared to those containing mismatches without gaps (Fig.

2-1, lanes 5, 6, and 7). Heteroduplexes migrate slower than their corresponding homoduplexes due to the "openness" in the double helix. In this study, it was observed that the heteroduplexes containing a single-base gap (Fig. 2-1, lane 8) migrated faster than those containing two-base mismatches (Fig 2-1, lane 5). This is most likely due to a larger opening created by the absence of a nucleotide on one strand than by two mismatched bases opposing one another. Previous studies also indicated that the greater retardation of electrophoretic mobility caused by insertions/deletions, relative to base substitutions, was due to a kink in the double helix required to accommodate the extra bases in the DNA heteroduplex (Bhattacharyya and Lilley, 1989; Wang and Griffith, 1991). When the heteroduplexes contained the same number of base differences, the migration of heteroduplexes containing mismatches with gaps (Fig. 2-1, lanes 11 to 17) was faster than those containing only mismatches (Fig. 2-1, lanes 3 to 7), but slower than those containing only gaps in our analysis of phytoplasma DNAs (Fig. 2-1, lanes 8 to 10, 18).

In previous studies, the heteroduplexes, formed between DNA fragments amplified from HIV_1 strains with mismatched nucleotides in absence of gaps, were detected under nondenaturing conditions when the degree of divergence exceeded about 1.4% (Delwart *et al.*, 1993, 1994). The results in this study showed that divergence in the 16S rDNA of phytoplasma isolates was detected at a level of as low as 0.2% by HMA, indicating that the sensitivity of HMA for detecting mutation in phytoplasma 16S rDNA is about seven-fold higher than in HIV. Previous studies (Zhong and Hiruki, 1994; Ceranic-Zagorac and Hiruki, 1996) indicated that closely-related phytoplasma isolates such as tomato stolbur (France) and eastern aster yellows (New York) phytoplasma isolates could be distinguished with high sensitivity by HMA but not by RFLP. Therefore, HMA provides an important means to differentiate closely-related phytoplasma isolates when other methods such as RFLP are not readily applicable.

HMA is an ideal method for characterizing and classifying phytoplasmas. At the meetings in Bordeaux (1994) and Orlando (1996), the Working Team on Phytoplasmas of the International Research Programme of Comparative Mycoplasmology of the International Organization for Mycoplasmology proposed that a threshold of 2.5% differences in the 16S rDNA sequence homology be used as a guide for establishing a new taxonomic group. In certain larger groups such as the AY group, finer differentiation of phytoplasma subclades was possible when their levels of 16S rDNA sequence homology differed by 1.2% (Seemüller *et al.*, 1998). Our results indicated that a divergence of 16S rDNA sequences of as low as 0.2% was detectable by HMA when a suitable reference DNA was used. In addition, it must be emphasized that heteroduplex mobility is proportional to the degree of DNA divergence (Delwart *et al.*, 1994). Therefore, when one considers that HMA not only differentiates the closely-related phytoplasma isolates with high sensitivity, but also recognizes a proportional relationship of heteroduplex mobility to the degree of DNA divergence, HMA stands as an ideal means for identification and classification of phytoplasmas.

While RFLP analyses of PCR amplified 16S rDNA have contributed to the construction of a phylogenetic tree of phytoplasmas, RFLP is based on the presence or absence of restriction sites in a DNA fragment and many restriction enzymes must be used to analyze the 16S rDNA for classification of phytoplasmas (Lee *et al.*, 1993; Gundersen *et al.*, 1994, 1996). The results of RFLP analyses may erroneously increase the distance of closely-related phytoplasma isolates or fail to detect significant differences between two phytoplasma strains. Certain phytoplasmas sharing very high levels of sequence homology may be classified into different groups. For instance, a pathologically dubious stone fruit strain ACLR exhibits levels of sequence homology of about 99% with the typical AY strains (Seemüller *et al.*, 1994). However, the ACLR strain was assigned to the group II since it differs by one *Alu*I site and one *Rsa*I site from typical AY strains (Schneider *et al.*, 1993; Gundersen *et al.*, 1996). On the other hand, two phytoplasma strains sharing low levels of sequence homology may be classified into one group. For example, a stolbur strain STOL shares about 97% DNA homology with typical AY strains (Seemüller, 1994) and was assigned to the same subgroup Ia since they share the same *Alu*I and *Rsa*I restriction profiles (Schneider *et al.*, 1993).

On the other hand, HMA is based on the principle that DNA heteroduplexes formed between different single nucleotide strands have a reduced mobility, in polyacrylamide gels under nondenaturing conditions, that is proportional to their degree of divergence (Delwart *et al.*, 1993). Certain differences between closely-related phytoplasma isolates were detected by HMA but not by RFLP analysis in this study and elsewhere (Ceranic-

Zagorac and Hiruki, 1996). Therefore, HMA provides an important means in phytoplasma differentiation and classification.

Some of the phytoplasmal diseases are caused by more than one phytoplasma strain. Previously, a mixed infection of phytoplasmas was detected by RFLP analysis and group-specific primers (Lee *et al.*, 1994; Alma *et al.*, 1996). However, to design group-specific primers, group specific sequence data must be known. To verify the group-specific primers, many phytoplasma strains from each group must be tested. In this study, the ability of HMA was examined to detect a mixed infection by two different phytoplasma strains. Six heteroduplex bands could be observed for a two DNA fragment mixture by HMA when a suitable DNA reference was used. The mixed DNA fragments could be easily identified by HMA. Therefore, with its ability to differentiate closely-related phytoplasmas with high sensitivity, HMA is a highly-efficient method for detecting mixed phytoplasma infections.

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Table 2-1. Oligonucleotide sequences used for 16S rDNA mutations

Oligonucleotide	Complementary sequence	Differences with L1
L1-1: 5' TGACCGAGTA 3'	L1-2: 5' TACTCGGTCA 3'	0
L2-1: 5' TGACGGAGTA 3'	L2-2: 5' TACTCCGTCA 3'	A single base
L3-1: 5' TGAGGGAGTA 3'	L3-2: 5' TACTCCCTCA 3'	Two bases
L4-1: 5' TGAGGCAGTA 3'	L4-2: 5' TACTGCCTCA 3'	Three bases
L5-1: 5' TGCGGCAGTA 3'	L5-2: 5' TACTGCCGCA 3'	Four bases
L6-1: 5' TCACCGAGAA 3'	L6-2: 5' TTCTCGGTGA 3'	Two single bases
L7-1: 5' TGACCAGTA 3'	L7-2: 5' TACTGGTCA 3'	One base deletion
L8-1: 5' TGACAGTA 3'	L8-2: 5' TACTGTCA 3'	Two base deletion
L9-1: 5' TGACGTA 3'	L9-2: 5' TACGTCA 3'	Three base deletion

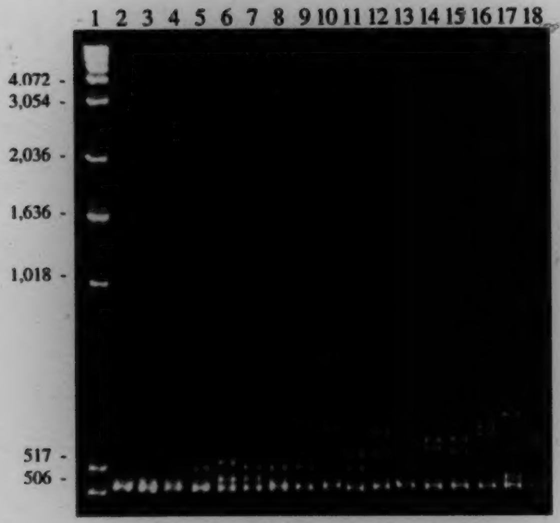

Fig. 2-1. HMA analysis of the mutated 16S rDNA fragments derived from AY27 phytoplasma. Lane 1, 1kb DNA maker; lane 2, DNA reference; lane 3, 1 bp mismatch; lane4, 2 bp mismatches at two different sites; lanes 5 to 7, 2 to 4 bp mismatches at a single site; lanes 8 to 10, 1 to 3 bp gaps; lane 11, 4 bp mismatches plus 1 bp gap; lane 12, 3 bp mismatches with 1 bp gap plus 1 bp gap; lane 13, 2 bp mismatches with 1 bp gap plus 2 single-base-pair gaps; lanes 14, 15, 7 bp mismatches with different base component; lane 16, 8 bp mismatches; lane 17, 6 bp mismatch plus 1 bp mismatch; lane 18, 10 bp gap.

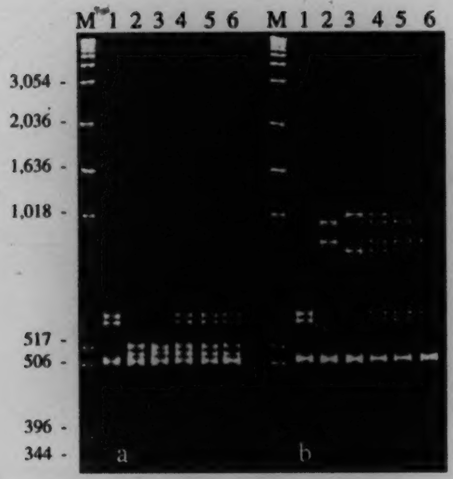

Fig. 2-2. HMA analysis of mixed DNA fragments in 5% polyacrylamide gel. (a): M, 1kb DNA marker, lane 1, combination of DNA fragments amplified from mutants L1-10 and L4-6; lane 2, combination of DNA fragments amplified from mutant L1-10 and clone L5-23; lane 3, combination of DNA fragments amplified from mutant L4-6 and L5-23; lane 4, 5, 6, combination of three DNA fragments amplified from mutants L1-10, L4-6 and clone L5-23 in ratio 1:1:1, 2:1:1, 5:1:1 respectively; (b) M, 1kb DNA marker; lane 1, combination of DNA fragments amplified from mutants L1-10 and L4-6; lane 2, combination of DNA fragments amplified from mutant L1-10 and clone L5-9; lane 3, combination of DNA fragments amplified from mutant L4-6 and L5-9; lane 4, 5, 6 combination of three DNA fragments amplified from mutants L1-10, L4-6 and clone L5-9 in ratio 1:1:1, 2:1:1, 5:1:1 respectively.

III

Heteroduplex Mobility Assay (HMA) as a Simple but Efficient Method for Identification and Classification of Phytoplasmas*

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* A version of this chapter was presented as an invitational seminar at the 13th International Congress of the International Organization for Mycoplasmology, July 13-19, 2000, Fukuoka, Japan.

3.1 Introduction

Accurate identification and classification of phytoplasmas will provide valuable information for studies on the epidemiology and control of phytoplasma diseases important to agriculture, horticulture, and forestry. However, the inability to culture phytoplasmas has made it difficult to characterize these pathogens. Historically, potential phytoplasma diseases have been detected mainly by observation of phytoplasma cells in plant phloem tissues by electron microscopy (Chen and Hiruki, 1975). Identification and classification of phytoplasmas were mainly based on host range and symptoms induced in natural hosts and on the specificity of their transmission by insect vectors (Chiykowski and Sinha, 1989). This dependence on biological and pathogenic characteristics in the classification of phytoplasmas has caused confusion in the naming and differentiation of these organisms, since the phytoplasma-induced biological properties alone were not sufficient for identification.

With the introduction of serological and nucleic acid hybridization methods, a number of polyclonal antisera (Sinha and Benhamou, 1983; Kirkpatrick and Garrott, 1984; da Rocha *et al.*, 1986), monoclonal antibodies (Lin and Chen, 1985, 1986; Shen and Lin, 1993), and DNA probes derived from phytoplasma genomic DNA (Kirkpatrick *et al.*, 1987; Lee *et al.*, 1988; Deng and Hiruki, 1990b; Kuske *et al.*, 1991) have been produced to detect and differentiate the organisms. The techniques have significantly improved phytoplasma identification and made it possible to classify phytoplasmas on the basis of DNA-DNA homology and serological data. Several organisms have been differentiated using these methods. However, these techniques are restricted by the limited number of probes and by the fact that undefined DNA fragments have been used as probes. The polygenetic positions of phytoplasmas in relation to each other and to other microorganisms cannot be identified by these techniques.

Since the introduction of polymerase chain reaction (PCR) to phytoplasma studies (Deng and Hiruki, 1990a), restriction fragment length polymorphism (RFLP) analyses of PCR-amplified highly-conserved genes have been used extensively to identify and classify phytoplasmas worldwide. However, the RFLP-based classification of phytoplasmas in certain groups does not fully coincide with the phylogenetic relationships of the organisms (Seemüller *et al.*, 1994) due to the nature of RFLP as examined in Chapter 2. Therefore, different RFLP patterns do not necessarily indicate significant phylogenetic distances, while in some cases similar RFLP profiles do not necessarily mean that the phytoplasma strains are closely related.

Heteroduplex mobility assay (HMA) is based on the principle that DNA heteroduplexes formed between related sequences reduce their mobilities in polyacrylamide gels, and that the degree of their delay is proportional to the extent of DNA divergence. Genetic relationships between multiple viral DNA template molecules can be rapidly evaluated. The sensitivity of HMA for DNA mutation detection is higher than any other techniques available, except for exhaustive DNA sequencing studies (Delwart *et al.*, 1993, 1994). HMA has been applied to the phytoplasma studies to analyze the relationships of closely-related phytoplasmas and the dynamic nature of phytoplasmas in different geographical regions (Zhong and Hiruki, 1994; Cousin *et al.*, 1998).

The objectives of this study were to evaluate the capability of HMA for classification of phytoplasmas by comparing with RFLP and DNA sequence analyses, and to select a suitable gene for the classification of phytoplasmas by HMA.

3.2 Materials and Methods

3.2.1 Source of phytoplasma isolates

In this study, 24 representative phytoplasma strains in 11 major groups with known 16S rRNA gene sequences and certain strains with known 16/23S spacer gene sequences were received from different sources in North America, Europe and Asia. The sources and abbreviations of phytoplasmas, and the accession numbers for sequences of 16S rRNA gene and 16/23S spacer region in GenBank (National Center for Biotechnology Information) are listed in Table 1. Aster yellows phytoplasma isolates 27 (AY27) was originally collected from the field in Alberta and maintained in periwinkle (*Catharanthus roseus* [L.] G. Don). Alfalfa witches'-broom (AWB), clover proliferation (CP), and potato witches'-broom (PWB) were collected from northern Alberta and maintained in the greenhouse. The following 13 phytoplasma strains were kindly provided as DNA samples by Dr. E. Seemüller, Biologische Bundesanstalt, Institut für Pflanzenschutz im Obstbau, Dossenheim, Germany: picris echioides phyllody (PEP), stolbur of pepper

(STOL), echium vulgare yellows (EVY), sunn hemp witches'-broom (SUNH), peach yellow leaf roll (PYLR), brinjal little leaf (BLL), grapevine yellows (VK), aster yellows from apricot (AYA, formerly ACLR), ash yellows (AshY), European stone fruit yellows (ESFY), pear decline (PD), phytoplasma from leafhopper *Psammotettix cephalotes* (BVK), apple proliferation (AT). The DNA samples of Tsuwabuki witches'-broom (TWB) and rice yellow dwarf (RYD) were the gifts of Dr. S. Namba, University of Tokyo, Tokyo, Japan. Flavescence dorée (FD) was kindly provided by Dr. E. Boudon-Padieu, Recherches sur les Phytoplasmes, INRA, Dijon, France. Elm yellows (EY) and Canadian X-disease (CX) phytoplasma strains in periwinkle were kindly provided by Dr. I.-M. Lee, Agricultural Research Service, USDA, MD, USA. The periwinkle plants infected with tomato big bud (TBB) or faba bean phyllody (FBP) were provided by Dr. M.T. Cousin, INRA, Station de Pathologie Végétale, Versailles, France.

3.2.2 PCR amplification

For the phytoplasma disease samples, total nucleic acids were extracted from about 1g of freshly-cut midrib tissues, as described previously (Wang *et al.*, 1998). The final pellets were dissolved in 50 µl of TE (10 mM Tris, pH 8.0; 1 mM EDTA) buffer and diluted to a final concentration of about 20 ng of nucleic acid per microliter with sterile deionized water.

Several primer pairs were selected in this study for PCR amplification of the partial 16S rRNA gene, entire 16S rRNA gene, 16/23S spacer region, and the large DNA fragment comprising the entire 16S rRNA and 16/23S spacer region from various phytoplasmas. The universal primer pair R16F2n/R2 (Lee *et al.*, 1993; Gundersen and Lee, 1996), designed on the basis of the 16S rRNA gene sequence of a Michigan AY strain, was used to amplify the partial 16S rRNA gene of about 1,240 bp in length from various phytoplasmas. The primer pair P1/P6 (Deng and Hiruki, 1990a) was employed to amplify the entire 16S rRNA gene while the primers P3/P7 (Kirkpatrick *et al.*, 1994; Smart *et al.*, 1996), located at the 3' end of 16S rRNA gene and 5' end of 23S rRNA gene respectively, were used for amplification of the 16/23S spacer region. The primers P1/P7 were employed to amplify the large DNA fragment comprising the entire 16S rRNA gene and 16/23S spacer region.

PCR-amplifications were performed in a 50 µl PCR reaction mixture as described previously (Wang *et al.*, 1998), with 200 µM each deoxynucleoside triphosphate (dNTP), 1.0 µM each primer, 1.25 U of DNA polymerase (Perkin-Elmer, Branchurg, NJ), and 50 ng of template DNA. The PCR was carried out for 35 cycles in an automated Thermal Cycler 2400 (Perkin-Elmer Cetus, Norwalk, CT). For the primer pairs R16F2n/R2, P1/P6, and P1/P7, the following conditions were used: denaturation at 94°C for 1 min (3 min for the first cycle), annealing at 55°C for 1 min, and extension at 72°C for 2-3 min. For primers P3/P7, the following conditions were employed: denaturation at 94°C for 40 sec (2 min for the first cycle), annealing at 55°C for 45 sec, and extension at 72°C for 50 sec. The final extension step was 10 min at 72°C and the reaction mixture was then held at 4°C. Five microliters of the PCR products were subjected to electrophoresis in a 1% agarose gel, and DNA bands were stained in ethidium bromide and visualized with a UV transilluminator.

3.2.3 Heteroduplex mobility assay (HMA)

To form DNA heteroduplexes between two phytoplasma strains, the PCR products were combined in equal volume, and subjected to denaturation and reannealing. In this study, an aliquot of 4.5 µl of the PCR products amplified from a phytoplasma reference was combined with each 4.5 µl of the PCR products amplified from various phytoplasma strains by the same primers. Then 1 µl of 10× annealing buffer (100 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 1 M NaCl) was added. One drop of mineral oil was overlaid on the reaction mixture. The mixture was subjected to heat denaturation in a water bath at 98°C for 4 min. Then the DNA fragments were hybridized by rapid cooling on ice. The hybridization products were separated by electrophoresis in a 5% polyacrylamide gel in 1× TBE buffer (0.088 M tris-borate, 0.089 M boric acid, 0.002 M EDTA) at 200-250V for 3-5 h (depending on the sizes of DNA fragments) at room temperature in PROTEAN II (Bio-Rad, Hercules, CA). DNA bands were stained in ethidium bromide and visualized with a UV transilluminator as above.

3.2.4 DNA sequencing

The 16/23S spacer region was sequenced for the phytoplasmas whose sequences were not available in GenBank data library. PCR-amplified DNA fragments were subjected to electrophoreses in 1% low melting point agarose gel. The corresponding DNA bands were cut and purified by the Agarose Gel DNA Extraction Kit (Boehringer Mannheim). The purified PCR product was directly used as a template for sequencing the 16/23S spacer region from both ends with primers P3 and P7 respectively by using the Dye Terminator Cycle Sequencing Core Kit (Perkin Elmer Cetus, Norwalk, CT) following the manufacturer's instructions.

3.2.5 Nucleotide sequence accession numbers

The nucleotide sequences of the 16S/23S spacer regions of the 14 phytoplasmas that were sequenced in this section will be released to the GenBank data library. The nucleotide sequences of the 16S rRNA gene for all phytoplasma strains except for AY27 and PWB and the 16/23S spacer region for the 10 phytoplasma strains used in this study can be found in the GenBank data library using the accession numbers as listed in Table 3-1.

3.3 Results

3.3.1 HMA analysis of the partial 16S rRNA gene

Heteroduplex bands were observed in a 5% polyacrylamide gel when the partial 16S rDNA (1.2 kb) amplified from AY27 was combined with each of those amplified from the rest of the phytoplasmas using the primers R16F2n/R2 (Fig. 3-1). Seven groups were differentiated among 24 phytoplasma isolates on the basis of heteroduplex mobilities. HMA classified groups such as AY, AP, SCWL, and AshY were in agreement with the results derived from the entire 16S rRNA sequence analysis (Seemüller *et al.*, 1998). However, certain groups derived by 16S rRNA sequence analysis were not differentiated by HMA. For example, heteroduplexes derived from CX and WX phytoplasmas showed similar migration patterns as those from the members of the AP group derived from sequence analysis, whereas the phytoplasmas in the CP and EY groups derived from sequence analysis showed similar heteroduplex mobilities. In addition, the phytoplasmas in the IAWB, FBP and BVK groups derived from sequence analysis also showed similar heteroduplex mobilities. On the other hand, different HMA profiles were observed among the members of certain groups. For example, in the X-disease group, TWB phytoplasma showed HMA profiles different from WX and CX phytoplasmas, while the heteroduplexes derived from phytoplasma RYD migrated slower than those from phytoplasma BVK in the sugarcane white leaf group.

3.3.2 HMA analysis of the entire 16S rRNA gene sequences

When the 16S rDNA fragments (about 1.5 kb) amplified from various phytoplasmas by PCR using primers P1 and P6 were subjected to HMA analysis using AY27 as a reference, eight groups, including the AY, STOL, FBP, PPWB, SCWL, AshY, CP and EY groups derived from 16S rRNA sequence analysis, were identified by HMA (Fig. 3-2). However, the phytoplasmas in the AP group derived from sequence analysis showed mobilities similar to CX, WX phytoplasmas, while PEP showed mobility similar to those of members of the FBP groups derived from sequence analysis. In addition, more genetic diversities were observed among the members of the FBP and CP group by HMA. Although phytoplasma isolates FBP, TBB, and SUNH were assigned to the same major group (FBP) by sequence analysis of 16S rRNA gene, the HMA profiles of the 16S rDNA fragments (about 1.5 kb) amplified from these phytoplasmas were different among them. The members of the CP group, BLL, CP and PWB, displayed different heteroduplex mobilities. In addition, similar to the results obtained by HMA analysis of the partial 16S rDNA fragments, HMA profiles of the full 16S rDNA fragments showed that phytoplasma TWB was different from WX and CX when AY27 was used as a reference.

3.3.3 HMA analysis of the 16/23S spacer region sequences

Ten major groups, derived from sequence analysis of the full 16S rRNA gene, were clearly discerned when the 16/23S spacer region amplified from the 24 representative phytoplasma strains were analyzed by HMA using AY27 as a reference (Fig. 3-3). Slight differences in the 16/23S spacer region were observed among the members of certain major groups, such as the AY, AP, WX, SCWL, CP, and FBP groups. However, the phytoplasmas STOL and SA in the STOL group, EY and FD in the EY group, and CX and WX in the X-disease group displayed very similar HMA profiles of the 16/23S spacer region.

Different phytoplasmas such as FD and PEP also were used as references for classification of phytoplasmas. When PEP phytoplasma was used as a reference, the phytoplasmas in the CP, WX, SCWL, and AP groups showed very similar heteroduplex mobilities (Fig, 3-4). Only slight differences of heteroduplex mobilities were observed among phytoplasmas in the WX, STOL, and AP groups when FD was used as a reference (Fig. 3-5). Therefore, these two phytoplasmas were not suitable as references for classification of phytoplasmas using HMA.

3.3.4 HMA analysis of the large DNA fragments

When the large DNA fragments comprising the full 16S rRNA gene and 16/23S spacer region were subjected to HMA analysis, 10 major groups resulted from HMA using AY27 as a reference (Fig. 3-6). PEP phytoplasma produced the HMA profiles similar to those yielded by the members of the FBP group, while the phytoplasmas in the STOL and AP groups showed similar heteroduplex mobilities. Compared with the results derived from HMA analysis of the partial 16S rDNA, the large DNA fragments displayed less divergence of HMA profiles among the members of the FBP group.

3.3.5 Correlation between heteroduplex mobilities and DNA distances

The values of heteroduplex mobilities were plotted against DNA distances, as shown in Figure 3-7. Heteroduplex mobilities were calculated as the average distance of migration of the heteroduplex bands divided by the average distance of migration of the homoduplex DNA bands. The DNA distances between different phytoplasmas were determined by the Kimura two-parameter equation (transversions weighted two times transitions) (Kimura, 1980). The relationship between relative mobilities and DNA distances were calculated by regression. The results indicated that the following equation best supported the data: $\text{mobility} = 0.1769 - 0.2346*\ln(\text{distance})$. The test for the regression coefficient indicated that the probability to accept the null hypothesis (mobilities and DNA distances are not related) is less than 0.0001. Therefore, the null hypothesis is rejected and, thus, the relationship between mobilities and DNA distances is significant at 99% confidence level.

3.4 Discussion

Nearly 300 phytoplasma isolates have been identified by serological methods and/or DNA-based analyses of the highly-conserved genes or random cloned DNA fragments from phytoplasma genomes (Lee *et al.*, 1998; Seemüller *et al.*, 1998). However, the classification of the phytoplasmas derived from sequence analysis and RFLP analysis of the highly-conserved genes was not always congruent. Therefore, the phytoplasmas used in this study were not selected randomly from phytoplasma populations but based on the following criteria: 1) the phytoplasmas were representatives of the major groups and caused diseases in economically important plant species; 2) the molecular data such as sequence and RFLP analyses of 16S rRNA gene were available for evaluation; 3) the taxonomic positions of the phytoplasmas may have differed between the sequence and RFLP analyses. The last two criteria were very important to evaluate HMA for the differentiation and classification of phytoplasmas.

Comparing the HMA profiles of each gene used in this study for classification of phytoplasmas, it was found that the 16/23S spacer region is the best choice for classification of phytoplasmas by HMA using AY27 as a reference. The 16/23S spacer region is located between the 3' end of the 16S rRNA gene and the 5' end of the 23S rRNA gene and contains a highly-conserved $tRNA^{Ile}$ gene. The 24 phytoplasma isolates clearly were classified into ten major groups that fully agreed with the results derived from sequence analysis of the 16S rRNA gene (Chapter 6). Slight differences among members in a given group can be used for classification of the phytoplasmas into different subgroups. In addition, the 16/23S spacer region is relatively short (about 300 bp in length) and can be easily amplified by PCR from a broad range of phytoplasmas, and thus can be used to analyze phytoplasmas at the same level. The heteroduplexes formed between the spacer regions amplified from different phytoplasmas can be easily and quickly separated from homoduplexes in a polyacrylamide gel. Furthermore, the 16/23S spacer region is more variable than the 16S rRNA gene and thus has been used to differentiate closely-related phytoplasmas (Kirkpatrick *et al.*, 1994; Smart *et al.*, 1996; Wang and Hiruki, 2001).

On the basis of analyses of the selected phytoplasmas, it has been demonstrated, for the first time, that HMA is a rapid, reliable, cost-efficient, and analytical method for not only differentiation, but also for classification of phytoplasmas when suitable genes are used. Phytoplasmas can easily be grouped by HMA profiles without using a large number of restriction endonucleases like RFLP analysis, and complicated procedures and high cost like DNA sequencing. Twenty-four phytoplasmas were classified into 10 groups by HMA analysis of the 16S rRNA gene and/or 16/23S spacer region. The conclusion was fully supported by sequence analysis of the 16S rRNA genes (Chapter 6; Seemüller *et al.*, 1998). HMA was also used for the classification of phytoplasmas in a few groups in previous studies (Zhong and Hiruki, 1994; Ceranic-Zagorac and Hiruki, 1996).

HMA is more accurate than RFLP analysis for classification of phytoplasmas. RFLP may fail to detect significant sequence differences between phytoplasmas. For example, certain phytoplasma strains, such as STOL and AY that share about 97% sequence homology of 16S rRNA gene, shared the identical RFLP patterns and were assigned into the same major group by RFLP (Schneider *et al.*, 1993). However, these phytoplasmas can be easily differentiated by HMA, as indicated in this study. On the other hand, the phytoplasmas that have high-level sequence homology may be assigned into different major groups by RFLP. In addition to the examples given in Chapter 2, ESFY phytoplasma and the members of the AP group share at least 98.4% sequence homology. However, the ESFY phytoplasma was assigned previously to a different group on the basis of an *Rsa*I restriction pattern difference in RFLP of the 16S rDNA (Schneider *et al.*, 1993). In contrast, the results in this study indicated that the ESFY phytoplasma belongs to the AP group. This conclusion was fully supported by sequence analysis (Seemüller *et al.*, 1994, 1998). The high sensitivity and accuracy of HMA also have been reported in previous studies (Zhong and Hiruki, 1994; Cousin *et al.*, 1998; Wang and Hiruki, 1999, 2000). Therefore, HMA provides superior precision for the differentiation and classification of phytoplasmas over any other methods except for DNA sequencing.

The PEP phytoplasma could be assigned to the FBP group. HMA profiles of the PEP and the members of the FBP group shared similar profiles when the partial 16S rRNA gene, full 16S rRNA gene, 16/23S spacer gene and the large DNA fragments were analyzed by HMA using AY27 as a reference. Sequence analysis of the 16S rRNA gene indicated that the average sequence homology among the members of the FBP group is 98.2%, while there was an average of 97.7% sequence homology between PEP and members of the FBP group. This value is higher than the threshold of 97.5% which allows for the separation of phytoplasmas into different major groups as established by the Working Team on Phytoplasmas of the International Research Programme of Comparative Mycoplasmology of the International Organization for Mycoplasmology. It follows, then, that the PEP and the other members of the FBP group may be assigned to different subgroups in the same major group (FBP) considering the unique HMA profile shown by each.

The correlation between heteroduplex mobility and DNA distance (calculated by the Kimura two-parameter equation) in this study was derived from HMA and sequence data. Use of this equation provides an estimate of the relationship between mobility and DNA distance. Estimated DNA distance values were not reliable in a range below 0.03 and above 0.35 which represent minimum and maximum limits. The difference between this equation and Delwart's equation (1993) may be due to the different genes in different classes of pathogens. The retardation of heteroduplexes in a polyacrylamide gel is not only related to the numbers, but also to the base compositions, the base pairs in mismatches and gaps, as well as the base compositions of flanking sequences. It is known that the genomes of phytoplasmas contain low G+C content (23.0 to 26.2 mol %) (Kollar and Seemüller, 1989). Sequence analysis of 16S rRNA gene also revealed that the rate of transversion substitutions was higher than that of transition substitutions, and most transversion substitutions are from purine to pyrimidine. All of these factors may affect the mobilities of heteroduplexes formed between different genes or DNA [illegible]gments from genomes of different organisms.

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Table 3-1. Phytoplasma strains used in this study

Abbreviation	Phytoplasma strain	Taxonomic group	Country	GenBank accession no.	
				16S rRNA gene	16/23S spacer
AY27	Aster yellows	Aster yellows	Canada	N/A	N/A
AYA	Aster yellows from apricot	Aster yellows	Spain	X68338	N/A
STOL	Stolbur of pepper	Stolbur	Serbia	X76427	AF035361
VK	Grapevine yellows	Stolbur	Italy	X76428	AF035362
PD	Pear decline	Apple proliferation	Germany	X76425	U54989
PYLR	Peach yellow leaf roll	Apple proliferation	USA	Y16394	U54990
AT	Apple proliferation	Apple proliferation	Italy	X68375	U54985
ESFY	European stone fruit yellows	Apple proliferation	Germany	X68374	U54988
CX	Canadian peach X-disease	X-disease	Canada	L33733	N/A
WX	Western X-disease	X-disease	USA	L04682	N/A
TWB	Tsuwabuki witches'-broom	X-disease	Japan	D12570	N/A
PEP	Picris echioides phyllody	Italian alfalfa WB	Italy	Y16393	N/A
FBP	Faba bean phyllody	Faba bean phyllody	Sudan	X83432	N/A
TBB	Tomato big bud	Faba bean phyllody	Australia	Y08173	AJ250822
SUNHP	Sunn hemp witches'-broom	Faba bean phyllody	Tailand	X76433	N/A
EVY	Echium vulgare yellows	Faba bean phyllody	Italy	Y16389	N/A
BVK	From leafhopper *P. cephalotes*	Sugarcane white leaf	Germany	X76429	N/A
RYD	Rice yellow dwarf	Sugarcane while leaf	Japan	D12581	N/A
AshY	Ash yellows	Ash yellows	USA	X68339	U54986
BLL	Brinjal little leaf	Clover proliferation	India	X83431	X83431
CP	Clover proliferation	Clover proliferation	Canada	L33761	N/A
PWB	Potato witches'-broom	Clover proliferation	Canada	N/A	N/A
EY	Elm yellows	Elm yellows	USA	L33763	U54991
FD	Flavescence dorée	Elm yellows	France	X76560	N/A

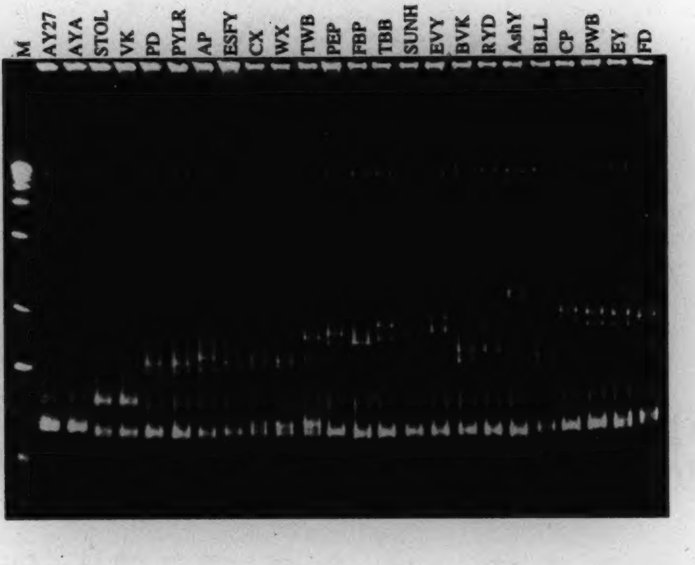

Figure 3-1. HMA of the partial 16S rDNA amplified from various phytoplasma isolates by PCR using primer pairs R16F2n/R2. Heteroduplexes and homoduplexes were separated by electrophoresis in a 5% polyacrylamide gel. AY27 phytoplasma was used as a reference. M, 1 kb DNA marker. The other abbreviations are the same as those described in Table 3-1.

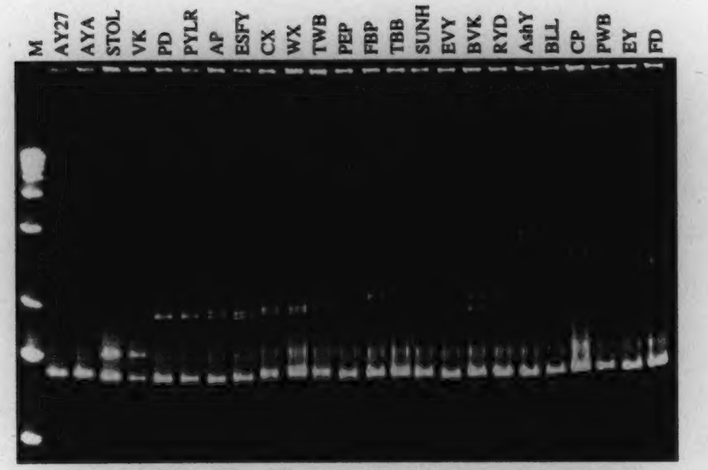

Figure 3-2. HMA of full 16S rDNA amplified from various phytoplasma isolates by PCR using primer pairs P1/P6. Heteroduplexes and homoduplexes were separated by electrophoresis in a 5% polyacrylamide gel. AY27 phytoplasma was used as a reference. M, 1 kb DNA marker. The other abbreviations are the same as those described in Table 3-1.

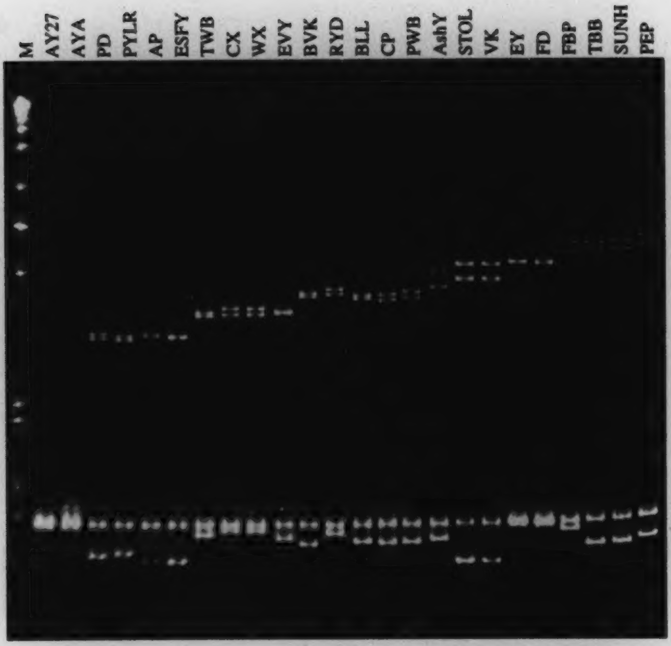

Figure 3-3. HMA of 16/23S spacer region amplified from various phytoplasma isolates by PCR using primer pairs P3/P7. Heteroduplexes and homoduplexes were separated by electrophoresis in a 5% polyacrylamide gel. AY27 phytoplasma was used as a reference. M, 1 kb DNA marker. The other abbreviations are the same as those described in Table 3-1.

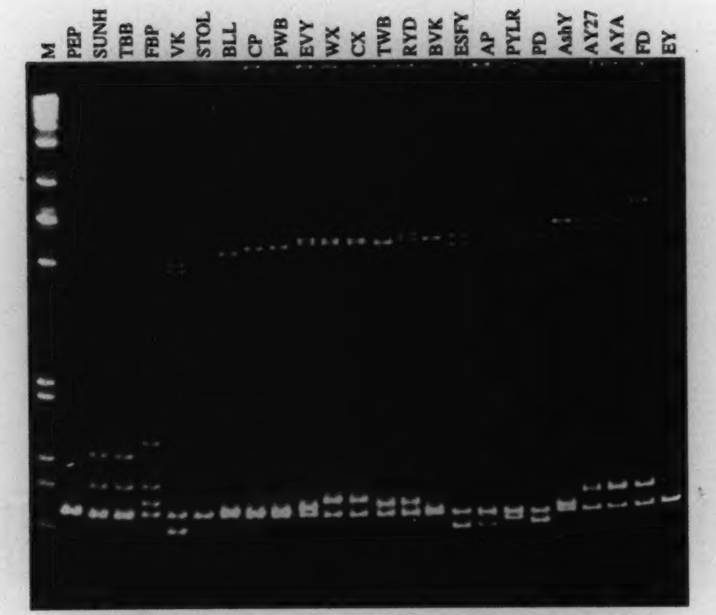

Figure 3-4. HMA of 16/23S spacer region amplified from various phytoplasma isolates by PCR using primer pairs P3/P7. Heteroduplexes and homoduplexes were separated by electrophoresis in a 5% polyacrylamide gel. PEP phytoplasma was used as a reference. M, 1 kb DNA marker. The other abbreviations are the same as those described in Table 3-1.

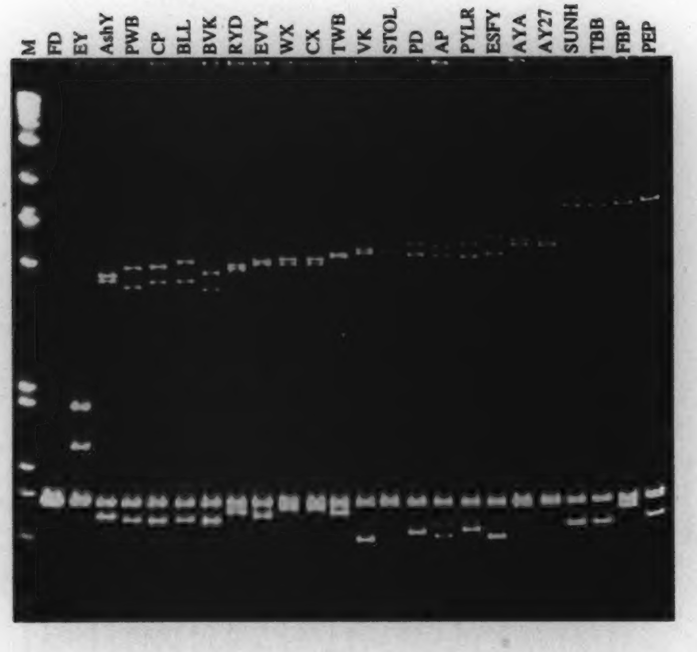

Figure 3-5. HMA of 16/23S spacer region amplified from various phytoplasma isolates by PCR using primer pairs P3/P7. Heteroduplexes and homoduplexes were separated by electrophoresis in a 5% polyacrylamide gel. FD phytoplasma was used as a reference. M, 1 kb DNA marker. The other abbreviations are the same as those described in Table 3-1.

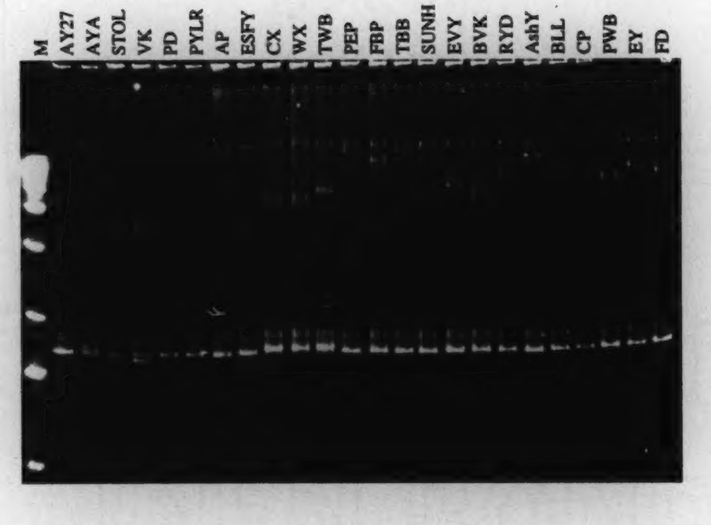

Figure 3-6. HMA of the large DNA fragment comprising the full 16S rDNA and 16/23S spacer region amplified from various phytoplasma isolates by PCR using primer pairs P1/P7. Heteroduplexes and homoduplexes were separated by electrophoresis in a 5% polyacrylamide gel. AY27 phytoplasma was used as a reference. M, 1 kb DNA marker. The other abbreviations are the same as those described in Table 3-1.

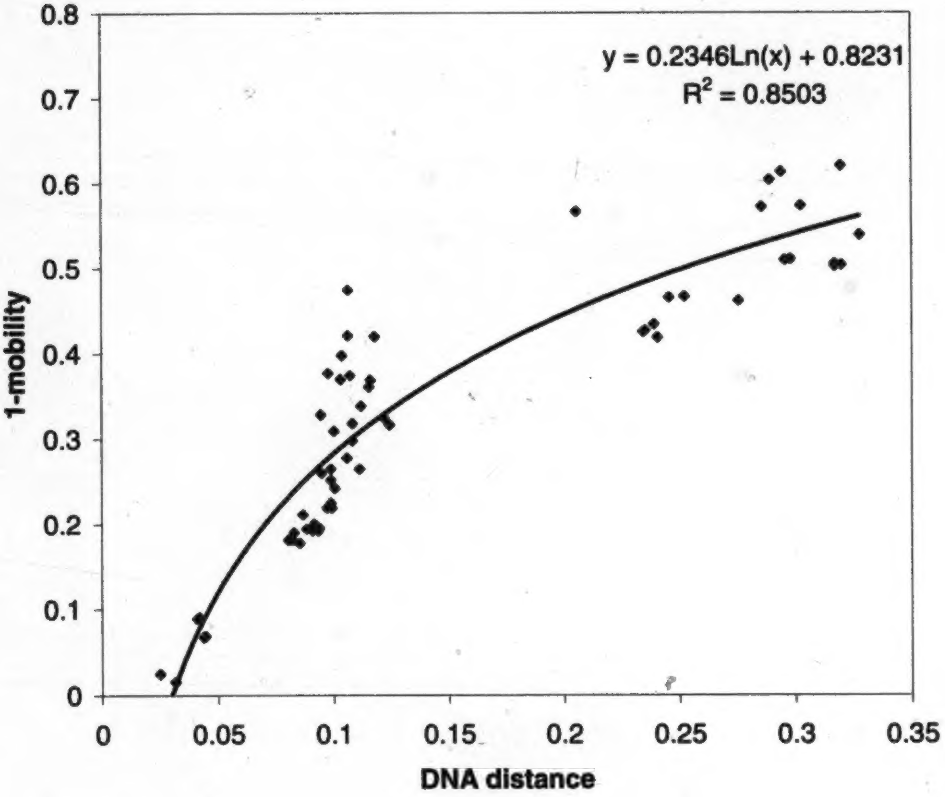

Figure 3-7. Relationship between DNA distance and mobility derived HMA analysis of 16S rRNA gene sequence of phytoplasmas.

IV

Use of Heteroduplex Mobility Assay (HMA) for Genetic Diversity Study of Phytoplasmas in the Clover Proliferation Group*

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* A version of this chapter has been published. Wang, K., and Hiruki, C. 2001. Phytopathology 91: 546-552.

4.1 Introduction

Clover proliferation (CP), potato witches'-broom (PWB), and alfalfa witches'-broom (AWB) are very important diseases and cause severe economic losses each year in Alberta. Since the association of CP with a phytoplasma was first reported (Chen and Hiruki, 1975), the investigation of their relationship has been carried out on the basis of nucleic acid hybridization with DNA probes randomly cloned from CP phytoplasma genomic DNA (Deng and Hiruki, 1991), polymerase chain reaction (PCR) with CP group-specific primers (Deng and Hiruki, 1990; Khadhair *et al.*, 1997), and restriction fragment length polymorphism (RFLP) analyses (Gundersen *et al.*, 1994; Lee *et al.*, 1991) of PCR-amplified highly-conserved genes. The above studies have revealed that AWB, CP, and PWB phytoplasmas are closely related to each other. This raises the question of whether AWB, CP, and PWB are caused by the same phytoplasma strain, since not all of the above techniques can differentiate among them. The answer to this question is important for understanding the epidemiology of these diseases and for disease management.

Heteroduplex mobility assay (HMA) is based on the principle that DNA heteroduplexes formed between related sequences have a reduced mobility, in polyacrylamide gels, which is proportional to their degree of divergence. Recently, HMA has been reported as a reliable means for identification and classification of phytoplasmas (Ceranic-Zagorac and Hiruki, 1996; Cousin *et al.*, 1998; Wang and Hiruki, 1999, 2000; Zhong and Hiruki, 1994). The sensitivity of HMA for detecting DNA mutation is higher than any other techniques currently available except for exhaustive DNA sequencing (Delwart *et al.*, 1993, 1994).

In this study, HMA analysis of the PCR-amplified 16/23S spacer region and 16S rRNA gene phytoplasma isolates AWB, CP, and PWB was employed to study genetic diversity of AWB, CP, and PWB phytoplasmas under field conditions. Two subgroups of AWB, CP, and PWB phytoplasmas were identified in field samples for the first time.

4.2 Materials and Methods

4.2.1 Sources of phytoplasmas

Aster yellows isolate 27 (AY27), CP, and PWB, originally collected from field-diseased plants in Alberta, were maintained in periwinkle [*Catharanthus roseus* (L.) Don] in the greenhouse and used as standard representative isolates. Field samples of AWB, CP, and PWB were also collected in northern Alberta and maintained in the greenhouse. Field phytoplasma isolates of AWB90 (hereafter numbers following alphabetical letters signify isolate number), and AWB92 were collected in 1990 and 1992, respectively; AWB1 to AWB8 were collected in 1998; all field isolates of CP and PWB were collected in 1996.

4.2.2 DNA extraction

Total nucleic acids were extracted from about 1g of freshly cut midrib tissues of diseased plants, as described previously (Wang *et al.*, 1998). The final pellets were dissolved in 50 µl of TE (10 mM Tris, pH 8.0; 1 mM EDTA) buffer and diluted to a final concentration of about 20 ng/µl of nucleic acid with sterile deionized water.

4.2.3 Primer pairs and PCR amplification

PCR assays were used for the detection of various phytoplasmas. The universal primers P1 (Deng and Hiruki, 1991) and P7 (Smart *et al.*, 1996) were previously designed on the basis of the 16S rDNA sequence of mollicutes, and were expected to amplify the entire 16S rRNA gene together with the 16/23S spacer region, a DNA fragment approximately 1,800 bp in length.

Amplifications were performed as described previously (Wang *et al.*, 1998), with 200 µM of each deoxynucleoside triphosphate (dNTP), 1.0 µM of each primer, 1.25 U of *Taq* DNA polymerase (Perkin-Elmer, Branchurg, NJ), and 50 ng of template DNA in a 50 µl reaction volume. Thirty-five PCR cycles were conducted in a DNA Thermal Cycler 2400 (Perkin-Elmer Cetus, Norwalk, CT) using the following conditions: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. The final extension step was 10 min at 72°C and the reaction mixture was held at 4°C. Five microliters of the PCR products were subjected to electrophoresis in an agarose gel, and DNA bands were stained in ethidium bromide and visualized with a UV transilluminator.

4.2.4 Nested PCR

To increase the sensitivity of PCR and to detect potential mixed infections, the primer pairs P3/P7 (Smart *et al.*, 1996) designed to amplify the 16/23S spacer region approximately 320 bp in length, and R16F2n/R2 (Gundersen and Lee, 1996; Lee *et al.*, 1993) designed to amplify the partial 16S rRNA gene approximately 1,200 bp in length, were used for nested PCR. PCR products amplified by the universal primers P1/P7 were diluted 0 to 100 times in sterile deionized water on the basis of the concentrations of amplified target DNA fragments and were used as templates for a subsequent series of 35 PCR cycles. PCR conditions for primers R16F2n/R2 were the same as above. The following parameters were used for primers P3/P7: denaturation at 94°C for 40s, annealing at 55°C for 45s, and extension at 72°C for 50s.

4.2.5 RFLP analysis of 16S rDNA sequences

The sensitivity of RFLP analysis in differentiating phytoplasma strains AWB, CP, and PWB was compared to HMA. The partial 16S rDNA sequences (1.2 kb) amplified by nested PCR using primers R16F2n/R2 were analyzed by restriction endonuclease digestion. Five microliters of each PCR product were digested with the following restriction endonucleases according to the instructions of the manufacturer: *Alu*I, *Hha*I, *Hpa*II, *Mse*I, *Rsa*I, and *Sau*3AI (GIBCO/BRL, Gaithersburg, MD). The restriction products were resolved on a vertical 5% polyacrylamide gel followed by staining in ethidium bromide. The DNA bands were then visualized using a UV transilluminator. The molecular weights of the fragments were determined by comparison with the 100 bp DNA ladder (BRL-life Technologies, Egenstein, Germany).

4.2.6 HMA analysis of 16/23S spacer region

The 16/23S spacer region and 16S rRNA gene amplified by nested PCR from AWB, CP, and PWB were analyzed by HMA respectively. A 4.5 µl aliquot of the PCR product amplified from AY27, used as a reference, was combined with 4.5 µl of each PCR product amplified from the various AWB, CP, and PWB isolates. Then 1 µl of 10× annealing buffer (100 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 1 M NaCl) was added. One drop of mineral oil was overlaid on the reaction mixture. After denaturing at 98°C for 4 min, the DNA fragments were hybridized on ice overnight. The hybridization products were separated by electrophoresis in a 5% polyacrylamide gel in 1× TBE buffer (0.088 M tris-borate, 0.089 M boric acid, 0.002 M EDTA) at 200-250V for 3-4 h at room temperature in PROTEAN II (Bio-Rad, Hercules, CA). DNA bands were stained in ethidium bromide and visualized under a UV transilluminator as above.

4.2.7 Nucleotide sequencing

PCR-amplified DNA fragments using primers P3/P7 were subjected to electrophoresis in a 1% low melting point agarose gel. The DNA band containing 16/23S spacer region was isolated from the gel and purified using the QIAquick Gel Extraction Kit (QIAGEN, Mississauga, Ontario, Canada) following the manufacturer's procedure. The purified DNA fragments were directly sequenced by using the DYEnamic™ ET Terminator Cycle Sequencing Premix Kit (Amersham Pharmacia Biotech, Inc., Cleveland, Ohio, USA). The primers P3 and P7 were used for cycle sequencing both strands of the 16/23S spacer region.

4.3 Results

4.3.1 Detection of phytoplasmas in the diseased samples

Direct PCR and nested PCR were employed to detect phytoplasmas of the field samples of diseased alfalfa, clover, and potato collected in Alberta from 1990 to 1998. For the direct PCR with universal primers P1/P7, approximately 1.8 kb DNA fragments were amplified from all the diseased samples. No DNA bands were observed from healthy ones. Following primary amplification, the PCR products were subjected to nested PCR amplification to increase the yield of PCR products for further analyses using primers P3/P7. DNA fragments approximately 320 bp in length were amplified from all diseased samples but not from the healthy one (Fig. 4-1).

4.3.2 Finer differentiation of CP phytoplasmas by HMA compared with RFLP analysis

To compare the sensitivity of HMA and RFLP in detecting genetic divergences of phytoplasma isolates collected from the field, the 16S rRNA gene amplified from AWB, CP, and PWB were analyzed by both HMA and RFLP. In the RFLP analysis of the 16S rRNA gene, the results repeatedly showed that the RFLP profiles of all field samples and standard isolates of AWB, CP, and PWB were identical for all restriction enzymes tested (Fig. 4-2). However, when the same PCR products (1.2 kb) were analyzed by HMA using AY27 as a reference, DNA heteroduplex bands were observed at approximately 1.8 kb position, in addition to the homoduplex bands at approximately 1.2 kb position (Fig. 4-3a). No heteroduplex band was observed for the combination of AY27-AY27. DNA heteroduplexes formed by the 16S rDNA fragment combinations of AY27 and each of AWB92, CP3, PWB, PWB1, PWB2, and PWB4 migrated faster than those formed by other combinations. The results indicated that the closely-related phytoplasmas were successfully differentiated by HMA but not by RFLP analysis.

The 16/23S spacer regions amplified from all phytoplasma isolates of AWB, CP, and PWB also were subjected to HMA using AY27 as a reference. After denaturation and re-annealing of the DNA fragments, heteroduplex bands were observed at the position approximately 900 bp for all AWB, CP, and PWB isolates but not for AY27. HMA profiles revealed that the heteroduplex mobilities of AWB92, CP3 and PWB, PWB1, PWB2, and PWB4 were identical, but were different from the remaining isolates of AWB, CP, and PWB (Fig. 4-3b). The results were consistent for HMA of both the 16/23S spacer region sequence and the partial 16S rRNA gene (Fig. 4-3a, 4-3b). Therefore, two subgroups of phytoplasma isolates of AWB, CP and PWB were identified on the basis of HMA profiles of the 16/23S spacer region and the 16S rRNA gene. Phytoplasma isolates AWB1-8, 90, CP, CP1-2, CP4-6, and PWB3, 5 were classified as subgroup I, while AWB92, CP3, PWB, PWB1, 2, 4 as subgroup II in the CP group. Since the 16/23S spacer regions of AY27 and AWB, CP, PWB were different in length, two homoduplex bands were observed for the 16/23S spacer genes. The homoduplex band at the position of approximately 320 bp represented the 16/23S spacer region of AY27, while the band at the position of approximately 300 bp represented those of AWB, CP, and PWB. The results suggested that each of the AWB, CP, and PWB diseases was caused by two different types of phytoplasmas in the CP group.

4.3.3 Nucleotide sequences of 16/23S spacer regions of AWB, CP, and PWB phytoplasmas

The approximately 265-nucleotide sequences of each 16/23 spacer region amplified from AWB, CP, and PWB were determined by directly sequencing both strands (Fig. 4-4). After making an alignment, the sequence data analysis revealed that while the members (AWB3, CP1, PWB3) of the subgroup I of the CP group shared an identical sequence in the 16/23S spacer region, the nucleotide sequences of the 16/23 spacer regions of phytoplasmas differed between subgroups I and II at positions 78, 99, and 237. The nucleotides G and T at the positions 78 and 237 of the spacer region in subgroup I phytoplasmas were substituted by A and C in subgroup II respectively, while a nucleotide G was inserted in the position 99 of the spacer region in subgroup II phytoplasma. The sequence data agreed with the results obtained by HMA.

4.4 Discussion

In this study, it was demonstrated that HMA was a highly sensitive diagnostic technique for the differentiation and identification of phytoplasmas. Twenty-three isolates of AWB, CP, and PWB were subjected to RFLP analysis. All isolates showed identical RFLP profiles with all endonucleases used in this study. Thus, RFLP failed to detect genetic differences among isolates of AWB, CP, and PWB. These results were consistent with previous studies in which AWB, CP, and PWB phytoplasmas were classified in the same phylogenetic group based on their identical RFLP profiles (Gundersen *et al.*, 1994; Khadhair and Hiruki, 1995; Khadhair *et al.*, 1997; Lee *et al.*, 1991, 1993, 1998). However, when HMA was applied to analyze the 16/23S spacer region or 16S rDNA amplified from the same phytoplasma isolates, the genetic differences were clearly discerned in both sequences. The results provided valid evidence that HMA is much more sensitive than RFLP analysis in differentiating the closely-related phytoplasma isolates. The high sensitivity of HMA in detecting DNA mutations of phytoplasmas also was demonstrated in previous studies in which a single base pair insertion/deletion or two base pair substitutions in a 500 bp DNA fragment were easily detected by HMA (Chapter 2; Wang and Hiruki, 1999, 2000).

In this study, two subgroups of AWB, CP and PWB were identified, for the first time, by HMA. Phytoplasmas of both types caused witches'-broom of alfalfa and potato plants, and proliferation of clover plants. However, the distributions of phytoplasmas in the two subgroups were uneven in AWB, CP, and PWB diseases. Nine of ten AWB samples, six of seven CP samples, and two of six PWB samples belonged to subgroup I. More samples are needed to detect phytoplasma strains common in each of AWB, CP, and PWB diseases. The differences between the two subgroups also were confirmed by sequence data in this study that showed a 3-base difference between subgroups I and II. Therefore, HMA provides a solid foundation to distinguish subgroups without labor-intensive RFLP analysis, and thus is highly suitable for epidemiological studies and future management of these diseases.

Our study clearly indicates that HMA is more accurate than RFLP analysis for the classification of phytoplasmas. While RFLP analysis of highly-conserved genes have been useful in constructing a phylogenetic tree of phytoplasmas, RFLP analysis relies on the presence or absence of restriction sites in a DNA fragment and, therefore, a set of restriction enzymes must be used (Gundersen, 1994, 1996; Lee *et al.*, 1993; 1998). DNA sequence analyses of more than 20 phytoplasma isolates in previous studies have indicated that RFLP-based classification of phytoplasmas in certain groups does not fully coincide with the phylogenetic relationships of the organisms (Kuske and Kirkpatrick, 1992; Seemüller *et al.*, 1994).

HMA provides an efficient method for the identification of phytoplasmas, and its use is promising especially for the survey of a large number of field samples and quarantine projects. HMA is highly capable of determining genetic distances between test samples and a standard phytoplasma. It is less expensive than RFLP analysis, which requires many expensive endonucleases. In this study, more than 20 phytoplasma isolates from field samples were classified into two subgroups by HMA. The results fully agree with the sequence data.

HMA offers an ideal method for studying the genetic diversity and molecular epidemiology of phytoplasmas. Very closely-related phytoplasmas such as AWB or CP showed genetic differences in the 16S rRNA gene and 16/23S spacer region between the different isolates that were collected in the same field from 1990 to 1998. Moreover, HMA has been demonstrated to be capable of detecting a single base-pair deletion/insertion or differentiating a single base-pair substitution in the 500 bp DNA fragments (Wang and Hiruki, 1999, 2000). Therefore, HMA may be used to monitor DNA sequence diversity of a given phytoplasma and to track the evolution of the phytoplasmas, so as to provide useful information for the study of phytoplasma disease epidemiology and potential disease control. A recent study on poplar witches'-broom by HMA has indicated that a phytoplasma isolate causing poplar witches'-broom in France was different from a German isolate, and thus suggested that the less severe symptoms caused by the German isolate may be correlated to the slight change in its nucleotide sequence (Cousin *et al.*, 1998).

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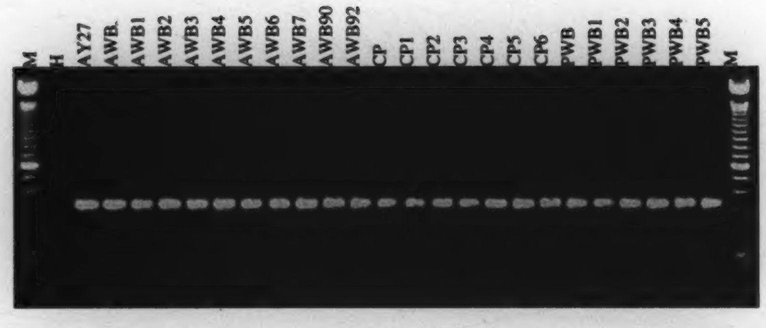

Figure 4-1. 16/23S spacer region of phytoplasmas amplified by nested PCR from various phytoplasma isolates using primer pair P3/P7 and separated by electrophoresis in a 1.5% agarose gel. M, 100 bp DNA ladder; H, healthy plant. Other abbreviations are the same as described in the text.

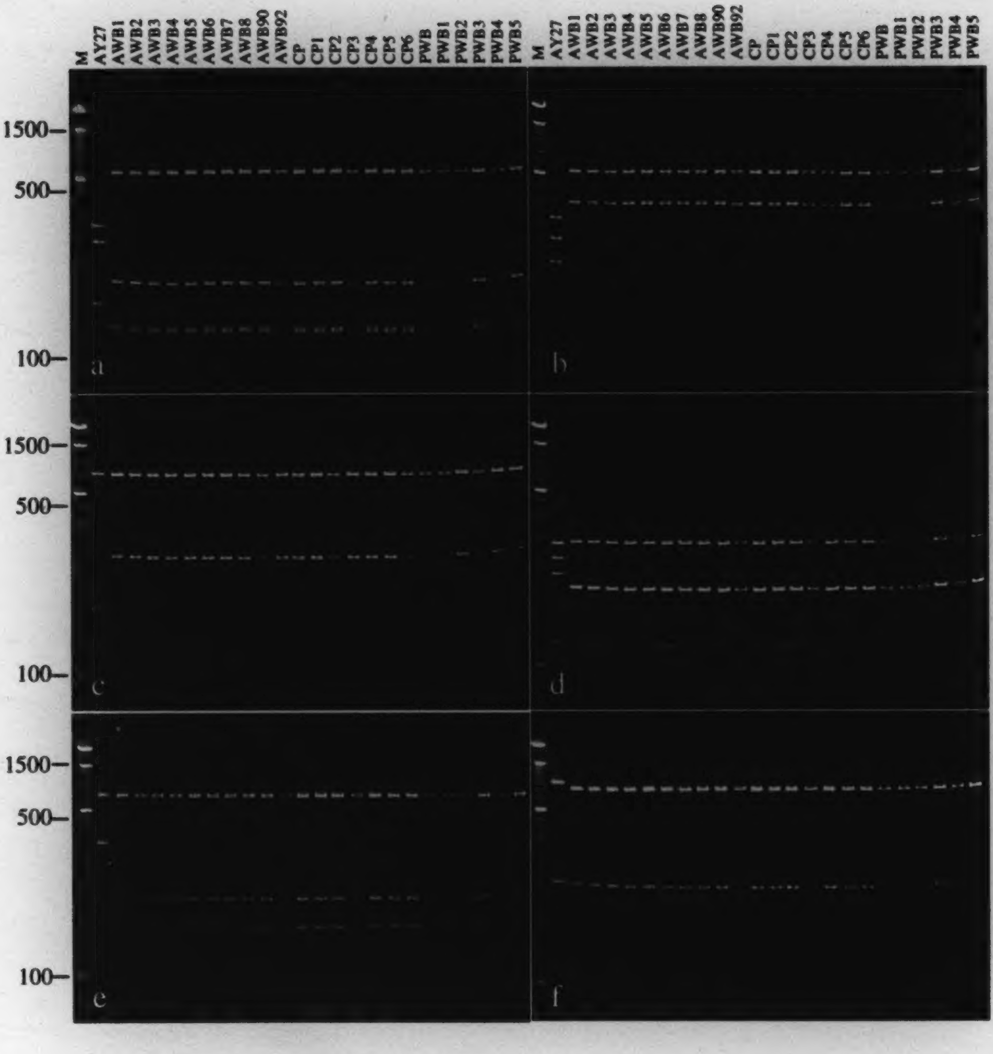

Figure 4-2. RFLP analysis of PCR-amplified 16S rDNA fragments from various CP, PWB and AWB phytoplasma isolates. PCR products were digested with restriction endonucleases *Alu*I (a), *Rsa*I (b), *Hpa*II (c), *Mse*I (d), *Sau*3AI (e), and *Hha*I (f). The restriction products were subjected to electrophoresis in a 5% polyacrylamide gel. M, 100 bp DNA ladder. Other abbreviations are the same as described in the text.

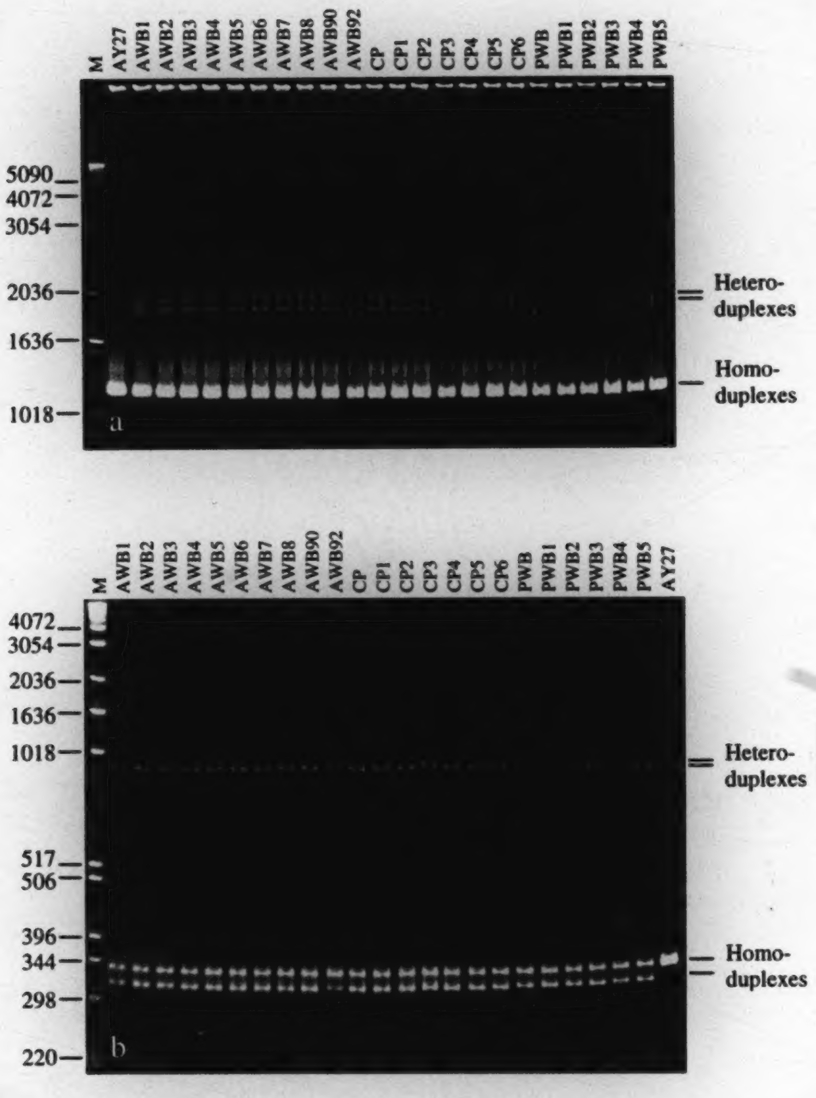

Figure 4-3. HMA analysis of nested PCR-amplified 16S rRNA gene (a) and 16/23S spacer region (b) from CP, PWB and AWB phytoplasma isolates. Heteroduplexes and homoduplexes were separated by electrophoresis in a 5% polyacrylamide gel. AY27 was used as a reference. M, 1 kb DNA ladder. Other abbreviations are the same as described in the text.

```
         1         11        21        31        41        51
AWB3     TCTAAGGACTATATATACTAAAATCATCATCTTCAGTTTTGAAAGACTTAAGTAGAAAAT
CP1      TCTAAGGACTATATATACTAAAATCATCATCTTCAGTTTTGAAAGACTTAAGTAGAAAAT
PWB3     TCTAAGGACTATATATACTAAAATCATCATCTTCAGTTTTGAAAGACTTAAGTAGAAAAT
AWB92    TCTAAGGACTATATATACTAAAATCATCATCTTCAGTTTTGAAAGACTTAAGTAGAAAAT
CP3      TCTAAGGACTATATATACTAAAATCATCATCTTCAGTTTTGAAAGACTTAAGTAGAAAAT
PWB4     TCTAAGGACTATATATACTAAAATCATCATCTTCAGTTTTGAAAGACTTAAGTAGAAAAT

         61        71        81        91        101       111
AWB3     AAGTTTTTCTTTTAAGCGCCAAAGGGCCTATAGCTCAG-TTGGTTAGAGCACACGCCTGA
CP1      AAGTTTTTCTTTTAAGCGCCAAAGGGCCTATAGCTCAG-TTGGTTAGAGCACACGCCTGA
PWB3     AAGTTTTTCTTTTAAGCGCCAAAGGGCCTATAGCTCAG-TTGGTTAGAGCACACGCCTGA
AWB92    AAGTTTTTCTTTTAAGCACCAAAGGGCCTATAGCTCAGGTTGGTTAGAGCACACGCCTGA
CP3      AAGTTTTTCTTTTAAGCACCAAAGGGCCTATAGCTCAGGTTGGTTAGAGCACACGCCTGA
PWB4     AAGTTTTTCTTTTAAGCACCAAAGGGCCTATAGCTCAGGTTGGTTAGAGCACACGCCTGA

         121       131       141       151       161       171
AWB3     TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAAAAAAGGTCCTGCTTAAA
CP1      TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAAAAAAGGTCCTGCTTAAA
PWB3     TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAAAAAAGGTCCTGCTTAAA
AWB92    TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAAAAAAGGTCCTGCTTAAA
CP3      TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAAAAAAGGTCCTGCTTAAA
PWB4     TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAAAAAAGGTCCTGCTTAAA

         181       191       201       211       221       231
AWB3     AAGTTCTTTGAAAAGTAGATAAACATGCTTTAAAATTTTCATAAAGTTGAAGGAAGTAAG
CP1      AAGTTCTTTGAAAAGTAGATAAACATGCTTTAAAATTTTCATAAAGTTGAAGGAAGTAAG
PWB3     AAGTTCTTTGAAAAGTAGATAAACATGCTTTAAAATTTTCATAAAGTTGAAGGAAGTAAG
AWB92    AAGTTCTTTGAAAAGTAGATAAACATGCTTTAAAATTTTCATAAAGTTGAAGGAAGCAAG
CP3      AAGTTCTTTGAAAAGTAGATAAACATGCTTTAAAATTTTCATAAAGTTGAAGGAAGCAAG
PWB4     AAGTTCTTTGAAAAGTAGATAAACATGCTTTAAAATTTTCATAAAGTTGAAGGAAGCAAG

         241       251       261
AWB3     GGCATATAGTGGATGCCTTGGCACT
CP1      GGCATATAGTGGATGCCTTGGCACT
PWB3     GGCATATAGTGGATGCCTTGGCACT
AWB92    GGCATATAGTGGATGCCTTGGCACT
CP3      GGCATATAGTGGATGCCTTGGCACT
PWB4     GGCATATAGTGGATGCCTTGGCACT
```

Fig. 4-4. Nucleotide sequences of 16/23S spacer regions of AWB, CP, and PWB phytoplasmas. The nucleotide sequences of primers P3, P7 are not included. Phytoplasma isolates of AWB3, CP1 and PWB3 were classified as members of the subgroup I while AWB92, CP3 and PWB4 are members of subgroup II in the CP group.

V

Phylogenetic Diversity of Phytoplasmas Based on Molecular Analysis of the 16/23S rRNA Spacer Regions by Heteroduplex Mobility Assay (HMA)

5.1 Introduction

Recently, the introduction of polymerase chain reaction (PCR) has made it possible to detect a broad range of phytoplasmas in infected plants and insect vectors (Deng and Hiruki, 1990; Lee, *et al.*, 1993; Gundersen *et al.*, 1996). On the basis of results from restriction fragment length polymorphism (RFLP) and sequence analyses of PCR-amplified 16S ribosome RNA (rRNA) genes, phytoplasmas have been classified into 20 major phylogenic groups or subclades. At present, RFLP analysis of PCR-amplified 16S rDNA is the routine method for differentiation and classification of phytoplasmas.

However, analyses of 16S rRNA gene may not always be capable of distinguishing closely-related phytoplasmas that differ in biological properties such as symptomatology and vector specificity, since the 16S rRNA gene is highly conserved during their evolution. The 16S rRNA gene alone does not reflect the full range of phenotypic diversity. Furthermore, the RFLP-based classification of phytoplasmas in certain groups does not fully coincide with the phylogenetic relationships of the organisms (Seemüller *et al.*, 1994) due to the nature of RFLP as discussed in Chapter 3. In addition, neither RFLP analysis nor sequence analysis are easily applicable to the characterization of a large number of field isolates of phytoplasmas due to their technical complexity and high cost. Therefore, more variable genes should be used for differentiation and classification of phytoplasmas by a simple, rapid and highly sensitive technique.

The spacer region (SR) located between 16S and 23S rRNA genes generally shows greater variation than the 16S rRNA gene since this region has fewer evolutionary constraints (Barry *et al.*, 1991). This region has been used to detect mycoplasmas as contaminants in cell cultures (Harasawa *et al*, 1993), as well as to identify subspecies of *Clavibacter michiganensis* (Li and De Boer, 1995). Sequence analysis of the 16/23S spacer region of more than 60 phytoplasma strains has resulted in a classification, which is similar to that derived from 16S rRNA data, with more detailed subdivision of phytoplasmas (Kirkpatrick *et al.*, 1994; Schneider *et al.*, 1995). The PCR using specific primers derived from this region of phytoplasmas have been an effective tool for the identification of particular phytoplasmas in field samples (Smart *et al.*, 1996). In addition, the 16/23S spacer region has been demonstrated to be the best choice for using heteroduplex mobility assay (HMA) to differentiate and classify phytoplasmas (Chapter 3).

HMA has a higher sensitivity for detecting DNA mutation than any other techniques currently available, except for exhaustive DNA sequencing (Delwart *et al.*, 1993, 1994). HMA in phytoplasma studies has been proven to be a rapid, sensitive and accurate means for identifying and classifying phytoplasmas, whereas other methods such as RFLP do not readily differentiate very closely-related phytoplasmas (Chapter 4; Zhong and Hiruki, 1994; Ceranic-Zagorac and Hiruki, 1996, Wang and Hiruki, 1999, 2000).

In Canada, several plant diseases associated with phytoplasmas cause severe economic loss to various vegetable, herb, tree or shrub industries (Hiruki and Wang, 1999; Hwang *et al.*, 1997; Khadhair *et al.*, 1998; Wang and Hiruki, 1998, 2001). Most of these phytoplasmas were identified as the members of the aster yellows and clover proliferation groups on the basis of DNA-based biotechnology or serological methods. Recently, many plant species showing symptoms resembling yellows or witches'-broom caused by phytoplasmas have been observed during field surveys. However, the causal agents of most of these diseases have not been identified and their genetic relationships to other pathogens have not been established.

Therefore, in this study, PCR was used to detect the pathogens using phytoplasma-specific primers, and HMA was employed to investigate genetic diversity of the pathogens using the 16/23S spacer gene as a taxonomic tool. The results revealed that these diseases were associated with phytoplasmas of wide genetic diversity. New phytoplasma strains were identified from known and new plant hosts found in Alberta.

5.2 Materials and Methods

5.2.1 Sources of phytoplasmas

Sixty-two phytoplasma isolates were used in this study and are listed in Table 1. Most of them were collected from natural host plants except for the following isolates that were transmitted to periwinkle (*Catharanthus roseus* (L.) G. Don) and maintained in the greenhouse: aster yellows isolate 27 (AY27), clover proliferation (CP), potato witches'-broom (PWB), tomato stolbur (STOL), Canadian X disease (CX), hydrangea aster yellows from Belgium (AYHB), hydrangea aster yellows from France (AYHF), elm yellows (EY), faba bean phyllody (FBP), and tomato big bud (TBB). The known phytoplasma strains AY27, CP, PWB, AWB, STOL, CX, EY, FBP, and TBB were used as references in the corresponding groups that were previously proposed on the basis of RFLP or sequence analyses of conserved genes (Gundersen *et al.*, 1996; Lee *et al.*, 1998; Seemüller *et al.*, 1998).

5.2.2 DNA Extraction

Approximately 1 g of fresh petioles, midribs or young symptomatic leaves was used for DNA extraction as previously described (Wang and Hiruki 1998). All DNA samples were treated by DNase free RNase and proteinase K. The final pellet was resuspended in 100 µl TE buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0) and stored at 4^oC until use.

5.2.3 Primers and PCR conditions

In this study, since most nucleic acids were extracted from phytoplasma-infected natural host plants which were collected at different times during growing seasons, it was difficult to amplify enough DNA fragments from some samples by direct PCR for further analyses. Therefore, nested PCR was used to amplify the 16/23S spacer region using two sets of primer pairs. The universal primers P1 (Deng and Hiruki, 1991) and P7 (Schneider *et al.*, 1995), designed to amplify the large DNA fragment comprising the entire 16S rRNA and 16/23S spacer region, were used as external primers. The primers of P3 and P7 (Schneider *et al.*, 1995) that are located at the 3' region of 16S rRNA gene and the 5' region of 23S rRNA gene were used as internal primers to amplify the 16/23S spacer region from various phytoplasma isolates. The oligonucleotide sequences of the primers used in this study are: P1, 5' AAGAGTTTGATCCTGGCTCAGGATT 3'; P3, 5' GGATGGATCA CCTCCTT 3'; P7, 5' CGTCCTTC ATCGGCTCTT 3'.

PCR amplification was performed in 100 µl reaction mixtures with 20 ng of total DNA, 1× PCR reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100), 2.5 mM MgCl, 2.5 U of *Taq* DNA polymerse (Perkin-Elmer Cetus, Norwalk, CT), 200 µM each dNTP, and 1 µM each primer. Forty PCR cycles were conducted in an automated thermocycler (Perkin-Elmer Cetus, Norwalk, CT). PCR amplification for primers P1/P7 was carried out under the following conditions: denaturation at 94°C for 1 min (3 min for the first cycle), annealing at 55°C for 1 min, and extension at 72°C for 2 min (10 min for the last cycle). The PCR products from the first amplification were undiluted or diluted up to 50 times, on the basis of the quantity of PCR products, using distilled deionized water and used as templates for nested PCR with primers P3/P7. The following conditions were used for nested PCR: denaturation at 94°C for 40 sec (2 min for the first cycle), annealing at 55°C for 45 sec, and extension at 72°C for 50 sec (10 min for the last cycle). Negative control containing DNA from healthy plants or no DNA template was used in each experiment. PCR products (5 μl) were analyzed by electrophoresis in a 1% agarose gel followed by staining in ethidium bromide and visualization of the DNA bands with a UV transilluminator.

5.2.4 HMA analysis of 16/23S spacer region sequences

To form DNA heteroduplexes, DNA fragments from different origins have to be combined, denatured, and reannealed. Each 4.5 μl of PCR products (1.2 kb) amplified from various phytoplasma isolates was combined with 4.5 μl of PCR products amplified from AY27 or CP respectively. To each combination, 1 μl of 10× annealing buffer (2 M NaCl, 100 mM Tris-HCl, pH 7.8, 20 mM EDTA) was added. The combined DNA fragments were denatured at 98°C for 4 min and hybridized on ice overnight. The reaction mixture was subjected to electrophoresis in a 5% polyacrylamide gel (acrylamide:bis 29:1) in TBE buffer at 200 V for 3 h at room temperature in Bio-Rad Protean II with a cooling system (Bio-Rad, Hercules, CA). The gel was stained in ethidium bromide and the DNA bands were visualized with a UV transilluminator.

5.3 Results

5.3.1 PCR amplification of phytoplasma 16/23S spacer region

When the primers P3/P7 were used for amplification of the phytoplasma 16/23S spacer region by direct PCR from nucleic acids extracted from various samples, approximately 250 bp DNA fragments were amplified from all DNA samples. However, weak bands were observed for the samples zinnia yellows, clarkia yellows, pot marigold yellows, French marigold yellows, willow witches'-broom, and monarda yellows.

Therefore, nested PCR was employed to amplify sufficient DNA fragments of 16/23S spacer region from these samples for further analysis. When the 1.8 kb PCR products amplified by primers P1/P7 were used as templates in nested PCR using P3/P7 as internal primers, sharp DNA bands approximately 250 bp in length were observed in an agarose gel for all samples from diseased plants, but not from healthy ones or the negative control. The results indicated that the titers of the phytoplasmas including ZinY, ClY, PMGY, FMGY, WWB and MonY in the infected plants were very low.

5.3.2 Classification of phytoplasmas by HMA analysis of 16/23S spacer region

The fragment of the spacer region amplified from AY27 was used as a reference and combined with those amplified from all phytoplasma samples. The DNA heteroduplexes were formed by pairwise combination of PCR-amplified fragments of the 16/23S spacer region from AY27 and each of the other phytoplasma isolates. The grouping of the tested phytoplasmas was clearly discerned by the profiles of HMA. A total of 62 phytoplasma isolates examined in this study were assigned to five major groups on the basis of HMA profiles of the 16/23S spacer region (Fig. 5-1). As explained in the following section, the AY group is the largest group including 48 phytoplasma isolates. Pin cherry witches'-broom (PCWB) phytoplasma was assigned to the X-disease (WX) group. Vinca phyllody (VP) phytoplasma produced HMA profiles similar to AWB, CP and PWB and also was classified in the CP group. The phytoplasmas associated with bamboo witches'-broom from China (BWB), and jujube witches'-broom from China (CJWB), Japan (JJWB) and Korea (KJWB) were very closely related to elm yellows phytoplasma and were classified into the EY group. Chokecherry witches'-broom (CWB) phytoplasma, similar to FBP and TBB phytoplasmas, was assigned to the FBP group by HMA analysis of the 16/23S spacer region. Similar results were obtained when CP was used as a reference (Fig. 5-2).

5.3.3 Finer differentiation of phytoplasmas the AY group

In the AY group, four subgroups were differentiated by HMA profiles among 48 phytoplasma isolates. When AY27 was used as a reference, the heteroduplexes, formed between 16/23S spacer groins from AY 27 and 29 phytoplasma isolates from the following plant species, co-migrated with their homoduplexes in a polyacrylamide gel:

asparagus (AspY), Belgium hydrangea (BHAY), California poppy (CaPY), canola (CanY1), carrot (CarY), celery (CelP), China aster (ChAY1), clarkia (ClY), coreopsis (CorY), cotton poplar (CPY), dahlia (DahY), dandelion (DanY1), French hydrangea (FHAY), French marigold (FMGY), Japanese paulownia (JPaWB), marigold (MGY), monarda (MonY1), pepper (PepY), petunia (PetY), potato (PPT), pyrethrum (PyrY), rudbeckia (RudY), scabiosa (ScaY), shellflower (ShY), spinach (SpY), strawflower (SFY), tagetes (TagY), tower poplar (TPY), and vinca (VinY). When CP phytoplasma was used as a reference to analyze the same sequence, these phytoplasmas showed HMA profiles identical to AY27. Since AY27 phytoplasma was identified as a typical strain in subgroup I-A on the basis of molecular data in previous studies (Lee *et al.*, 1993, 1998; Gundersen *et al.*, 1996), these phytoplasmas were assigned to subgroup I-A with AY27. A total of 15 phytoplasma isolates from the following plant species shared very similar HMA profiles of the 16/23S spacer region when AY27 or CP phytoplasma was used as a reference: callistephus (CalY), canola (CanY2), China aster (ChAY2), dictamnus (DicY), feverfew (FFY), giant imperial (GIY), monarda (MonY2), parsley (ParY), pot marigold (PMGY), purple coneflower (PCY), Queen Anne's lace (QALY), swan river daisy (SRDY), willow (WWB), winged everlasting (WEY), and zinnia (ZinY). However, the heteroduplexes formed between 16/23S spacer regions, amplified from AY27 and these phytoplasmas, migrated slower than the homoduplexes. These phytoplasmas also showed migration of heteroduplexes slower than AY27 when CP phytoplasma was used as a reference and thus were assigned in subgroup I-B. A phytoplasma isolate (DanY2) from dandelion yellows disease produced a unique HMA profile when the spacer region was analyzed by HMA using both references AY27 or CP. Therefore, this phytoplasma was assigned to a new subgroup I-C in the AY group. Paulownia witches'-broom from China (CPaWB) and Korea (KpaWB) yielded very similar HMA profiles to each other, but different from others, and thus were assigned into subgroup I-D in the AY group.

5.3.4 Genetic diversity of phytoplasmas

The genetic diversity of phytoplasmas was observed in phytoplasmas occurring in a wide range of plant species grown in Alberta. The phytoplasmas associated with monarda yellows and canola yellows showed different HMA profiles. The phytoplasmas MonY1 and CanY1 were assigned to subgroup I-A, while MonY2 and CanY2 were classified into subgroup I-B on the basis of HMA profile of the 16/23S spacer region using AY27 or CP as a reference. Similarly, dandelion yellows occurring in Alberta and paulownia witches'-broom diseases occurring in Asia were also associated with phytoplasmas of two different subgroups respectively: DanY1 from Edmonton, Alberta, belonged to subgroup I-A, while DanY2 from Grande Prairie, Alberta, belonged to I-C; PaWB from China and Korea belonged to subgroup I-D, while PaWB from Japan belonged to I-B.

5.4 Discussion

In this study, a total of 62 phytoplasmas were differentiated and classified into five major groups by HMA analysis of the 16/23S spacer region. Fifty phytoplasma isolates were identified as members of the AY group. For the first time, phytoplasma infections were reported on 23 plant species in Canada. This not only broadens our understanding of phytoplasma diseases, but also provides important information for phytoplasma disease management in Canada.

This study demonstrated that the phytoplasmas in subgroups I-A and B were prevalent in Alberta, and that more phytoplasmas were found in subgroup I-A than in subgroup I-B. In previous studies, only a limited number of phytoplasma diseases were reported to be associated with phytoplasmas in the AY group in Alberta (Hwang *et al.*, 1997; Khadhair *et al.*, 1998; Wang and Hiruki, 1998; 2001). The results in this study indicated that plant diseases caused by phytoplasmas in the AY group were more widespread than previously thought and were causing severe economic loss in many plant species, especially canola. The disease incidence of canola yellows has been rapidly increasing in recent years (Dr. Gary Stringam, personal communication). The fact that certain trees and weeds also are infected by AY phytoplasmas, and suffer from diseases such as willow witches'-broom and poplar yellows, suggests that they may serve as natural sources of phytoplasmas causing yellows diseases in agricultural and horticultural crops. To confirm this hypothesis, future studies need to identify insect vectors involved in phytoplasma transmission from diseased trees or weeds to agricultural and horticultural crops.

RFLP and sequence analyses of 16S rRNA gene of PaWB in previous studies showed that all PaWBs from China, Japan and Korea belonged to the same subgroup 16Sr I-D in the AY group (Lee *et al.*, 1993; Wang 1997). However, HMA profiles of 16/23S spacer region of a PaWB isolate from Japan were different from those from China and Korea. The Japanese PaWB isolate was a member of subgroup I-B, while the Chinese and Korean PaWB isolates were the members of subgroup I-D. This is the first report to show that PaWB disease is associated with genetically different phytoplasmas. In addition, it provides further evidence that the high sensitivity of HMA makes it possible to differentiate closely-related phytoplasmas by analyzing the less conserved 16/23 spacer region.

Witches'-broom disease of poplar was observed in Bulgaria (Atanasoff, 1973), Netherlands (Van der Meer, 1980), France (Sharma and Cousin, 1986) and Germany (Seemüller and Lederer, 1988) and was demonstrated to be associated with phytoplasmas that were differentiated into three subgroups in the AY group (Berges *et al.*, 1997; Cousin *et al.*, 1998). Recently, poplar witches'-broom disease was reported in Canada and identified as a member of the AY group (Hiruki and Wang, unpublished). This study further confirmed that phytoplasmas associated with both cotton and tower poplar witches'-broom were members of subgroup 16Sr I-A in the AY group by HMA analysis of the 16/23S spacer region.

It has been reported that two or more phytoplasmas associated with the same host plant could cause similar symptoms in different geographical regions. For example, phytoplasmas associated with tomato big bud diseases were determined to be members of the AY group in the United States (Lee *et al.*, 1992), the FBP group in Australia (Schneider *et al.*, 1995), and the WX group in Brasil (Boudon-Padieu *et al.*, 1996). In this study, HMA analysis of the 16/23S spacer region revealed that two distinct phytoplasmas associated with certain phytoplasma diseases in a single species such as dandelion, China aster, monarda, canola, and poplar, produced very similar symptoms. However, certain diseases in the same host plant caused by different phytoplasmas were found in the same area while no mixed infections were identified in this study. Therefore, two possibilities may be related to this phenomenon. One is that the cross protection observed in virus diseases may exist in phytoplasma diseases. The other is that the mixed infections of these phytoplasmas may occur (Lee *et al.*, 1994; Alma *et al.*, 1996), although they were not found in this study. Thus, further field study of these phytoplasma diseases and research on cross protection of phytoplasmas should be carried out in the future.

The mechanisms of diversity in phytoplasma pathogenicity remain unclear. For example, HMA analysis of the 16/23S spacer region in this study revealed that phytoplasmas in subgroup I-A are associated with diseases in 30 plant species, producing yellows or witches'-broom symptoms. Previous studies indicated that phytoplasmas in subgroups I-A and I-B could induce a wide variety of symptoms such as virescence, phyllody, small and faintly colored flowers, flower malformations, shortening or elongation of internodes, small and deformed leaves, yellowing, and decline (McCoy *et al.*, 1989).

A phytoplasma isolate (DanY2) from dandelion yellows disease was identified as a member of a new subgroup in the AY group on the basis of the unique HMA profile of the 16/23S spacer region. The results were in full agreement with the previous conclusion that dandelion yellows phytoplasma contained a piece of DNA fragment which was inserted into the 16S rDNA compared with AY27 phytoplasma on the basis of RFLP analysis of the 16S rRNA gene (Wang and Hiruki, 2001). Since this dandelion yellows phytoplasma isolate was collected from an alfalfa field where alfalfa witches'-broom disease was widespread, it is possible that the inserted DNA fragment may have originated from the AWB phytoplasma as the result of gene flow via insect vector(s). The insect vector feeds on AWB phytoplasma-infected alfalfa plants and acquires AWB phytoplasma. Thus, these two phytoplasmas may have a chance to exchange their genetic information in the genetic common pool, resulted in a new phytoplasma. When the insect vector feed on dandelion plants again, the new phytoplasma may multiply in dandelion plants and a new population of the phytoplasma may be established. This hypothesis also needs to be verified by future genetic studies of host-insect-phytoplasma interactions.

5.5 References

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Table 5-1. Phytoplasma isolates used in this study

Abbr.	Disease	Host plant	Origin
AWB	Alfalfa witches'-broom	*Medicago sativa* L.	Alberta
AspY	Asparagus yellows	*Asparagus officinalis* L.	Edmonton, AB
AY27	Aster yellows	*Callistephus chinensis* L.	Edmonton
BWB	Bamboo witches'-broom		China
CaPY	California poppy yellows	*Eschscholzia californica* Cham. cv. Tai Silk	Edmonton, AB
CalY	Callistephus yellows	*Callistephus chinensis* (L.) Nees	Edmonton, AB
CX	Canadian X disease	*Prunus persica* (L.) Batsch.	USA
CanY1	Canola yellows	*Brassia napus* L.	Edmonton, AB
CanY2	Canola yellows	*Brassia napus* L.	Edmonton, AB
CarY	Carrot yellows	*Daucus carota* L. cv. sativa	Alberta
CelP	Celery proliferation	*Apium graveolens* L.	Edmonton, AB
ChAY1	China aster yellows	*Callistephus chinensis* (L.) Nees	Edmonton, AB
ChAY2	China aster yellows	*Callistephus chinensis* (L.) Nees	Edmonton, AB
CWB	Chokecherry witches'-broom	*Prunus virginiana* L.	Edmonton, AB
ClY	Clarkia yellows	*Clarkia unguiculata* Lindl.	Edmonton, AB
CorY	Coreopsis yellows	*Toreopsis tinctoria* Nutt.	Edmonton, AB
CP	Clover proliferation	*Trifolium hybridum* L.	Canada
CPY	Cotton poplar yellows	*Populus* spp.	Brooks, AB
DahY	Dahlia yellows	*Dahlia* spp.	Edmonton, AB
DanY1	Dandelion yellows	*Taraxacum officinale* Weber	Edmonton, AB
DanY2	Dandelion yellows	*Taraxacum officinale* Weber	Grande Prairie, AB
DicY	Dictamnus albus yellows	*Dictamnus albus* L.	Edmonton, AB
EY	Elm yellows	*Ulmus* spp.	USA
FBP	Faba bean phyllody	*Vicia faba* L.	Sudan
FFY	Feverfew yellows	*Chrysanthemum parthenium* (L.) Bernh	Edmonton, AB
FMGY	French marigold yellows	*Tagetes patula* L.	Edmonton, AB
FHAY	Hydrangea aster yellows	*Hydrangea Macrophylla* Ser.	France
BHAY	Hydrangea aster yellows	*Hydrangea Macrophylla* Ser.	Belgium
CJWB	Jujube witches'-broom	*Zizyphus jujuba* Mill.	China
JJWB	Jujube witches'-broom	*Zizyphus jujuba* Mill.	Japan
KJWB	Jujube witches'-broom	*Zizyphus jujuba* Mill.	Korea
MGY	Marigold	*Tagetes patula* L.	Edmonton, AB

Table 5-1 (continue). Phytoplasma isolates used in this study

Abbr.	Disease	Host plant	Origin
MonY1	Monarda yellows	*Monarda fistulosa* L.	Brooks, AB
MonY2	Monarda yellows	*Monarda fistulosa* L.	Brooks, AB
ParY	Parsley yellows	*Petroselinum crispum* (Mill.) Nym.	Edmonton, AB
CPaWB	Paulownia witches’-broom	*Paulownia tomentosa* L.	China
JPaWB	Paulownia witches’-broom	*Paulownia tomentosa* L.	Japan
KPaWB	Paulownia witches’-broom	*Paulownia tomentosa* L.	Korea
PepY	Pepper yellows	*Capsicum annuum* L.	Brooks, AB
PetY	Petunia yellows	*Petunia grandiflora* Hort.	Edmonton, AB
PCWB	Pincherry witches'broom	*Prunus pensylvania*	Edmonton, AB
PMGY	Pot marigold yellows	*Calendula officinalis* L.	Edmonton, AB
PPT	Potato purple top	*Solanum tuberosum* L.	Brooks, AB
PWB	Potato witches’-broom	*Solanum tuberosum* L.	Alberta
PCY	Purple coneflower yellows	*Echinacea purpurea* (L.) Moench.	Brooks, AB
PyrY	Pyrethrum yellows	*Tanacetum cinerariaefolium* L.	Edmonton, AB
QALY	Queen Anne’s lace yellows	*Ammi majus* L.	Edmonton, AB
RudY	Rudbeckia yellows	*Rudbeckia fulgida* Ait.	Edmonton, AB
ScaY	Scabiosa yellows	*Scabiosa astropurpurea* L.	Edmonton, AB
GIY	Scabiosa yellows	*Scabiosa atropurpurea* L. cv. Giant Imperial	Edmonton, AB
ShY	Shellflower yellows	*Alpinia speciosa* (Wendl.) Schum.	Canada
SpY	Spinach yellows	*Spincia oleracea* L.	Edmonton, AB
SFY	Strawflower yellows	*Helichrysum bracteatum* L.	Edmonton, AB
SRDY	Swan river daisy yellows	*Brachycome mutifida* DC. Gglab. cv. Misty Pink	Edmonton, AB
TAGy	Tagetes yellows	*Tagetes patula* L.	Edmonton, AB
TBB	Tomato big bud	*Lycopersicon esculentum* Mill.	Australia
TPP	Tower poplar yellows	*Populus* spp.	Brooks, AB
VP	Vinca phyllody	*Catharanthus roseus* (L.) G. Don.	Canada
VinY	Vinca yellows	*Catharanthus roseus* (L.) G. Don.	Edmonton, AB
WWB	Willow witches’-broom	*Salix* spp.	Edmonton, AB
WEY	Winged everlasting yellows	*Ammobium alatum* L.	Edmonton, AB
ZinY	Zinnia yellows	*Zinnia elegans* Jacq. cv. Dahlia flower	Edmonton, AB

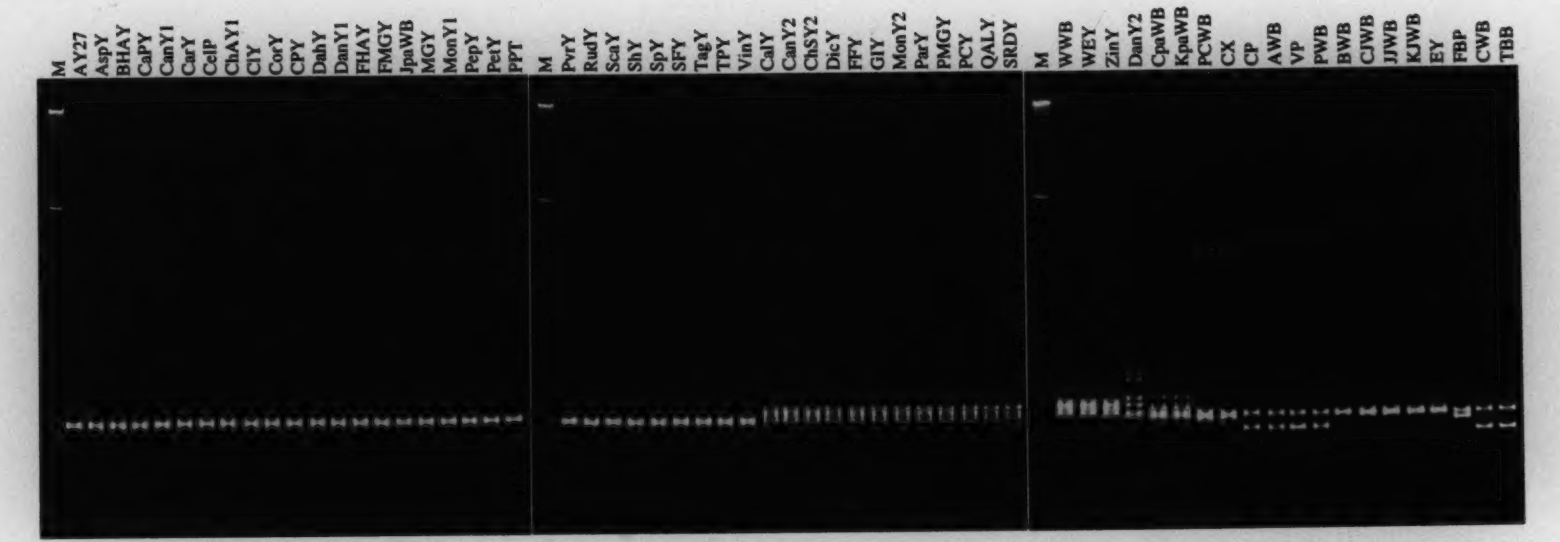

Figure 5-1. HMA of PCR-amplified 16/23S spacer region from various phytoplasma isolates. Heteroduplexes and homoduplexes were separated by electrophoresis in 5% polyacrylamide gels. AY27 phytoplasma was used as a reference. M, 1 kb DNA ladder. The other abbreviations are the same as those described in Table 5-1.

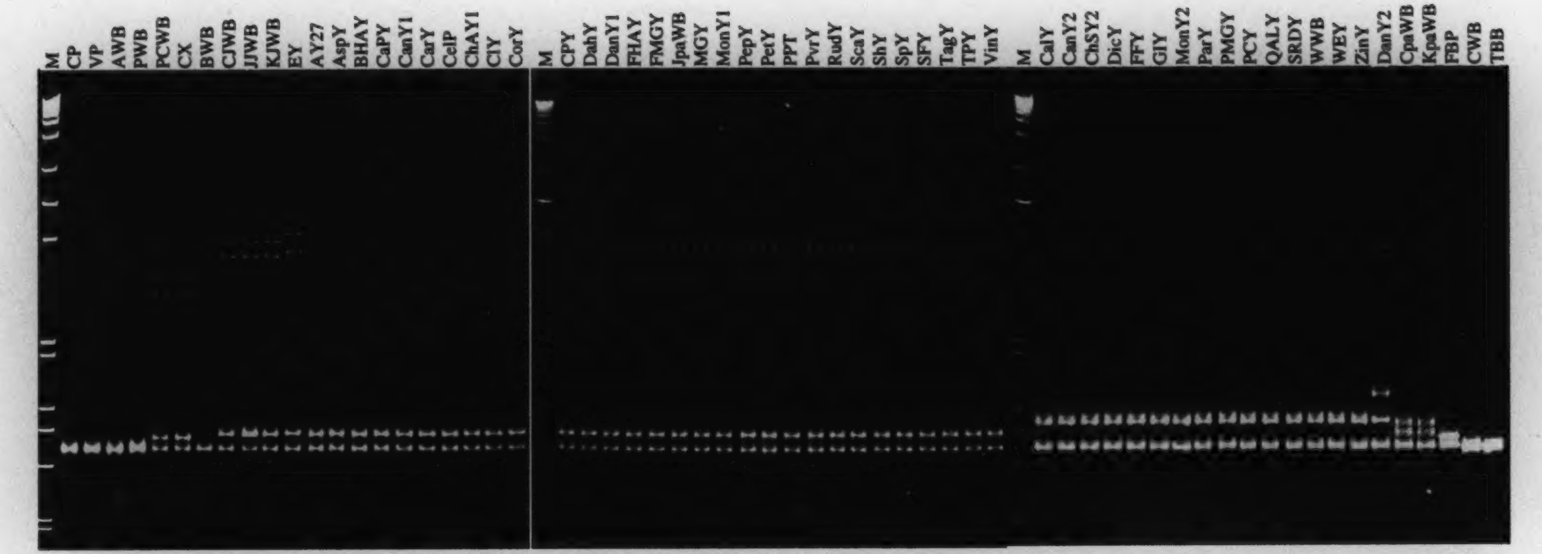

Figure 5-2. HMA of PCR-amplified 16/23S spacer region from various phytoplasma isolates. Heteroduplexes and homoduplexes were separated by electrophoresis in 5% polyacrylamide gels. CP phytoplasma was used as a reference. M, 1 kb DNA ladder. The other abbreviations are the same as those described in Table 5-1.

VI

Molecular Evolution of Phytoplasmas Based on Polymorphisms in the 16S rRNA Genes and the 16/23S Spacer Regions

6.1 Introduction

Ribosomal operons have paramount relevance to the study of bacterial evolution and phylogeny. The 16S rRNA gene is the most widely used molecular chronometer for inferring microbial phylogeny and has been instrumental in developing a comprehensive view of microbial phylogeny and systematics (Gutell *et al.*, 1994). In most prokaryotes, the ribosomal genes form an operon with the order 16S-23S-5S and are transcribed as a single polycistronic RNA that has to be processed to give rise to the RNA species present in the mature ribosome (Condon *et al.*, 1992). The organization of the mollicute rRNA genes generally follows the characteristic eubacterial order (Iwami *et al.*, 1984; Sawada *et al.*, 1984) and processing of rRNA gene transcripts (Muto *et al.*, 1992). The spacer between the 16S and 23S genes, called the intergenic spacer region (ISR), sometimes contains tRNA genes in gram-negative bacteria. Most mollicutes carry only one or two ribosomal operons while the number of ribosomal operons in bacteria is up to 11 (Gürtler and Stanisich, 1996). However, all phytoplasmas examined contain two ribosomal operons which appear to be identical to each other (Schneider and Seemüller, 1994). A single $tRNA^{Ile}$ gene is located in the spacer region of phytoplasmas (Kirkpatrick *et al.*, 1994) and acholeplasmas (Nakagawa *et al.*, 1992), while no tRNA genes could be found in the region in other members of the class *Mollicutes* (Razin, *et al.*, 1998).

The full-length or nearly full-length sequences of the 16S rRNA genes and 16/23S spacer regions from more than 60 phytoplasma strains have been determined (Namba *et al.*, 1993; Gundersen *et al.*, 1994; Kirkpatrick *et al.*, 1994; Seemüller *et al.*, 1998). The phylogenetic analyses of these sequences have delineated at least 20 subclades (groups) and indicated that phytoplasmas are more closely related to *Acholeplasma laidlawii* than to *Mycoplasma* species (Lim and Sears, 1992; Seemüller *et al.*, 1998). However, the characters of the secondary structure involved in the 16S rRNA genes and 16/23S spacer regions were not considered in the previous phylogenetic analyses. Secondary structure is one of the major features of ribosomal RNA genes. Mutations in the highly conserved hairpin loops or helices may cause changes related to the ribosome function through the influence of rRNA folding (Tapprich and Hill, 1986; Tapprich *et al.*, 1989). The details of secondary structure may serve as markers of the evolutionary process in certain lineages.

A wealth of data derived from sequence and RFLP analyses of the ribosomal RNA genes has been used to construct a phylogenetic tree of phytoplasmas. This has made it possible to clarify the relationship with other members of the class *Mollicutes* (Gundersen *et al.*, 1994; Lee *et al.*, 1993, 1998; Seemüller *et al.*, 1998). The objectives of this study were to investigate the molecular evolution of 16S rRNA genes and 16/23S spacer regions, and to make some inferences about molecular evolution of phytoplasmas from a phylogenetic tree constructed by aligning the sequences based on secondary structure.

6.2 Materials and Methods

6.2.1 Phytoplasma strains and sequence data

Twenty-four phytoplasma strains were selected from 11 major groups defined by the previous analysis of the 16S rRNA gene sequence (Seemüller *et al.*, 1998) as listed in Table 3-1 in Chapter 3. Except for aster yellows isolate 27 (AY27) and potato witches'-broom (PWB), the nucleotide sequences of the 16S rRNA genes of the selected phytoplasmas are available in GenBank (National Center for Biotechnology Information, Bethesda, Md.) under the accession numbers listed in Table 3-1. The 16/23S spacer region sequences of the following 11 phytoplasmas: apricot aster yellows (AYA), stolbur of pepper (STOL), grapevine yellows (VK), pear decline (PD), peach yellow leaf roll (PYLR), apple proliferation (AT), European stone fruit yellows (ESFY), tomato big bud (TBB), ash yellows (AshY), brinjal little leaf (BLL), and elm yellows (EY), are also available in GenBank. The nucleotide sequences of 16S rRNA genes or 16/23S spacer regions for the rest of the phytoplasmas that were unavailable were sequenced as described in Chapter 3.

6.2.2 Secondary structure modeling

The secondary-structure model of the 16S rRNA molecule from AY27 was constructed manually on the basis of the counterpart from *Escherichia coli* proposed by Woese etc. (1983) and distributed by Gutell (1994). The helices were given different numbers as proposed by Van de Peer etc. (1996b). The numbers with P indicated the helices specific to a prokaryotic model. The program *mfold*, version 3.1 (Zuker *et al.*, 1999; Mathews *et al.*, 199[illegible] was used to predict the folding of each helix. The foldings were calculated with default energy data settings at 37°C.

6.2.3 Sequence alignment and the phylogenetic tree

The sequences of both the full 16S rRNA gene and 16/23S spacer region from 24 phytoplasmas and *Acholeplasma laidlawii* (Weisburg *et al.*, 1989) were aligned respectively by using the program CLUSTAL W (Thompson *et al.*, 1994). The resulting alignments were visually inspected for logical placement of gaps and were manually adjusted, if necessary, on the basis of the conserved sequence patterns and secondary structure.

The genetic distances among the sequences were estimated by the HKY85 + Γ model by allowing transitions and transversions to occur at the rate of 2:1 with varied base frequencies and varied substitution rates among sites (Hasegawa *et al.*, 1985). A phylogenic tree was constructed by PAUP 4.0 beta version (Swofford, 2000) using a neighbor-joining method (Saitou and Nei, 1987) by implementing the tree bisection and reconnection branch-swapping algorithm to find the optimal phylogenetic tree(s). The branch lengths were estimated using the least squares algorithm (De Soete, 1983). One thousand bootstrap samples were analyzed to estimate the stability and to assess the confidence of nodes on the tree (Felsenstein, 1985). The phylogenetic tree was viewed as a rooted graphics using *Acholeplasma ladilawii* as an outgroup.

6.3 Results and Discussion

6.3.1 Secondary structure analysis

The secondary structure model of 16S rRNA from AY27 phytoplasma was constructed on the basis of the model of the 16S rRNA from *E. coli* (Gutell *et al.*, 1994). Five 'tertiary' interactions derived by Gutell *et al.* (1996) on the basis of coordinated substitutions also were indicated in Figure 6-1. Nine highly variable regions were identified in the following helices: 6, 8-12, 17-18, P23-1, 29, 37 and P37-1, 43, 45, 46, and 49. Of 308 polymorphisms across the 16S rRNA genes among the 23 phytoplasma strains, 225 polymorphisms were located in the highly variable regions. The highly-conserved sequences of the 16S rRNA were found mainly in single-stranded regions that may be specific rRNA-protein interactions or functional sites. Previous studies indicated that the highly-conserved hairpin loop of helix 27 was directly involved in ribosomal subunit association (Tapprich and Hill, 1986) and initiation of protein synthesis (Tapprich *et al.*, 1989). Meanwhile, another highly-conserved sequence, $GCCGCG_{525}$, which was found in all but one of the eubacterial catalogs, was variable in phytoplasmas, i.e. CCCGCG for CP phytoplasma, GCTGCG for PD, PYLR, AT and ESFY phytoplasmas. This sequence is essential for ribosomal function (Woese and Gutell, 1989; Powers and Noller, 1991). In addition, previous studies indicated that a single base mutation in rRNA genes could affect translation efficiency and cell growth (Triman *et al.*, 1998). Thus, certain unique sequences of phytoplasmal 16S rRNA gene might be closely linked to the inability of phytoplasmas to grow on growth media.

A total of 58 positions were involved in deletion/insertion mutations among the 23 phytoplasmas. The most deletion/insertion mutations occurred in the following helices: 6, 11, 45-46, and 49 that had 9, 5, 4, and 8 bases deletion/insertions respectively. Although a base in the stem could be deleted or inserted (such as a thymine that was inserted in the helix P23-1 for CP, BLL, EY, FD, and AshY phytoplasmas), most deletion/insertions occurred in or close to the hairpin loops or bulges. In addition, if a base was deleted or inserted in a stem, a compensatory deletion/insertion mutation in the opposite position was not observed and, except for helices 6 and 11, frequently resulted in the formation of a bulge. Therefore, these deleted/inserted bases may not be essential for the functions of the 16S rRNA gene of the phytoplasma, or may confer a specific function for a given phytoplasma. It is also very interesting to note that the substitutions took place when several bases were deleted from or inserted in a loop such as helices 6, 11.

A total of 250 substitution mutations were found in the 16S rRNA gene of 23 phytoplasmas. As a general rule, a compensating substitution in the opposite strand occurred when a base was substituted in a strand so as to stabilize the actual stem in the molecule, which is similar to the 16S rRNA gene from *E. coli* (Van de Peer *et al.*, 1996a). However, in mycoplasma studies (Pettersson *et al.*, 1996, 1998), a compensatory mutation in the corresponding nucleotide position was not observed for over 40 substitutions localized in the stem regions of the 16S rRNA molecule. The arrangement of elongation factors G and Tu of phytoplasmas was in contrast to that of mycoplasmas (Berg and Seemüller, 1999). Therefore, the substitution model also suggested that phytoplasmas were distinct from the genus *Mycoplasma*.

Noncanonical base pairing was found in the secondary structure of the 16S rRNA molecular from phytoplasmas. Most of these are G·U pairings. For example, a conserved U in one strand can have A or G as its counterparts while a preferred G in one strand can pair with C or U. In addition, C can tolerate A or G as a pairing nucleotide and a C·G base pair can be substituted with a C·A base pair such as nucleotide A at the position 1108 in STOL, VK, BLL, CP, and TWB phytoplasmas, while a A·U base pair can be substituted with a A·C pair such as T_{247} in TWB phytoplasma. The A·C pair might not necessarily affect the stability of a stem (Van de Peer, 1996) since X-ray analyses of DNA heteroduplexes found that the A·C pair contains a protonated C or a tautomeric configuration of A or C (Hunter *et al.*, 1986).

The 16/23S spacer regions containing a tRNA^{Ile} gene were highly variable among phytoplasmas. For the upstream and downstream of tRNA^{Ile} gene, nucleotide sequences in different sizes were deleted or inserted and resulted in extensive polymorphisms of the spacer region in phytoplasmas. It is very interesting to note that the sequences 5'-TCATCTTCAGTTTTGAA(or G)AGACTTA-3' upstream and 5'-TCTTTGAAAAGAT AAA-3' downstream of the tRNA^{Ile} gene are extremely conserved among all tested phytoplasmas in this study (Fig. 6-2). The deletion/insertion mutations only occurred between this highly-conserved sequence and tRNA^{Ile} or 16S or 23S rRNA genes. Therefore, these two highly-conserved sequences may be involved in the processing of rRNA and tRNA^{Ile} genes, or in other functions during rRNA transcription. The deletion/insertion areas in the spacer region are rich in A+T contents possibly due to AT-biased directional mutation pressure during the evolution of phytoplasmas. It was suggested that the members of mollicutes were subjected to this particular selection pressure which was in favor of A·T base pairs (Samuelsson and Borén, 1992). Under AT pressure, the nonfunctional or functionally least important areas may undergo a higher evolution rate from G·C to A·T than the functional areas since mutations in the nonfunctional areas may be neutral or less deleterious than mutations in other regions.

The secondary structure model of tRNA^{Ile} gene of phytoplasmas was constructed manually on the basis of the general tRNA secondary structure model (Fig. 6-3). The nucleotide sequences of the acceptor stem, D stem, anticodon stem and loop in the tRNA^{Ile} gene were highly conserved across 24 phytoplasmas. Only one substitution occurred in the D loop. The most variable region was the T stem and loop which contained five substitutions among 24 phytoplasmas in 10 major groups. Phytoplasmas (except for PEP) in the AY, AshY, CP, and FBP groups shared an identical sequence of tRNA^{Ile} gene, while the STOL and WX groups have one substitution in the D loop and the EY group has one substitution in the T stem compared to the AY group. All phytoplasmas in the AP group shared an identical nucleotide sequence for tRNA^{Ile} gene that contains two substitutions in the T stem. Phytoplasma BVK, RYD and PEP each has a unique nucleotide sequence. The only noncanonical base pairing found in tRNA^{Ile} gene is the G·U pairing which may have evolved from A·U or G·C pairing.

6.3.2 Classification of phytoplasmas

A phylogenetic tree was constructed by analysis of the full 16S rRNA gene sequences from diverse phytoplasmas using *Acholeplasma laidlawii* as an outgroup (Fig. 6-4). Ten major groups were defined among 23 phytoplasmas on the basis of branching patterns and by differences in the 16S rDNA sequence homology greater than 2.5%. To be consistent with a naming system in previous studies (Gundersen *et al.*, 1994; Seemüller *et al.*, 1998), these groups were delineated as follows (group members in brackets): aster yellows (AY) group (AY, AYA), stolbur (STOL) group (STOL, VK), apple proliferation (AP) group (AT, ESFY, PD, PYLR), faba bean phyllody (FBP) group (FBP, PEP, SUNH, TBB), X-disease (WX) group (CX, WX, TWB), pigeon pea witches'-broom (PPWB) group (EVY), sugarcane white leaf (SCWL) group (BVK, RYD), ash yellows (AshY) group (AshY), clover proliferation (CP) group (CP, BLL), and elm yellows (EY) group (EY, FD). Each group was supported by an extremely high bootstrap value. In previous studies, comparative analyses of the entire genome tree and gene trees generally indicated that the higher a bootstrap value, the more clades from the gene tree are on the genome tree (Commings *et al.*, 1995; Otto *et al.*, 1996), although the accuracy of a tree was also affected by other factors such as the number of characters and the number of taxa (Hillis and Bull, 1993). The clades not on the entire genome tree were frequently observed on a tree supported by low bootstrap values. Therefore, evolutionary conclusions must be based only on trees with high bootstrap values.

Except for PEP phytoplasma, the major groups delineated in this study agree with those derived from previous studies in which PEP phytoplasma was defined as a different group with Italian alfalfa witches'-broom (Seemüller *et al.*, 1998). The sequence similarities of the full 16S rRNA gene between PEP and TBB, SUNH, FBP are 98.1, 97.80, 97.87% respectively. DNA distances of the 16S rRNA gene between PEP and TBB, SUNH, FBP are 0.0189, 0.0224, 0.0217 respectively on the basis of the HKY85 + Γ model (Table 6-1). In 1994, the International Research Program of Comparative Mycoplasmology of the International Organization for Mycoplasmology set a threshold value of 2.5% to differentiate phytoplasma strains into different major groups. Therefore, the PEP phytoplasma is classified into the FPB group in this study. This conclusion is supported by the high bootstrap value. For finer differentiation, the members in the FBP group may be differentiated into three subgroups: I including TBB and SUNH, II including FBP, and III including PEP.

The topology of the tree constructed in this study is slightly different from those in previous studies (Gundersen *et al.*, 1994; Lee, *et al.*, 1998; Seemüller *et al.*, 1998). For example, the branch of the WX group is shown after the branch of the FBP group in this study, while they were shown as sister branches by Seemüller and co-workers (1998). Since a phylogenetic tree constructed by Seemüller and co-workers did not show the bootstrap value for each branch, the tree could not be evaluated. The branching order among the groups SCWL, PPWB, AshY, CP, and EY was uncertain as the bootstrap values were low (Gundersen *et al.*, 1994). The differences of the tree patterns may be due to different sequence alignments for phylogenetic analysis.

Sequence alignment is a critical early step in comparative molecular and evolutionary studies. In this study, the 16S rRNA gene sequences were initially aligned by the program CUSTAL W which produced more consistent alignments than other programs, such as Malign and PileUp, over the range of gap costs tested (Hickson *et al.*, 2000). However, the regions containing gaps still might not be aligned properly by the program. Therefore, after initial alignment by the program, the nucleotide sequences consisting of deletion/insertions, especially for the helices 6, 9, 11, and 49, were reexamined visually and adjusted manually, if necessary, on the basis of the secondary structure.

There are several models such as Jukes-Cantor model (Jukes and Cantor, 1969), Kimura's 2-parameter model (Kimura, 1980), and the Felsenstein model (Felsenstein, 1981), to estimate the genetic distance between two nucleotide sequences. In this study, the HKY85 + Γ model was selected to measure DNA distances (Table 6-1) as this model allows the bias of transition : transversion ratio on each site and unequal base frequencies as well as variation in rates of substitution among sites. The results derived from the HKY85 +Γ model may be close to the observed data. Comparative studies on the observed pattern of nucleotide substitutions between mtDNA sequences from humans and chimpanzees showed that the expected pattern under the HKY85 +Γ model matches the observed data better than other models (Tamura, 1994; Yang *et al.*, 1994). In nature, transitions accumulate rapidly and begin to reach saturation, whereas transversions are much more rare and appear to accumulate linearly with time (Page and Holmes, 1998). Sequence analyses indicated that transition: transversion ratios were 9.0, 1.75, and 2.7 for mtDNA, 12S rRNA, and pseudo-globin genes respectively (Wakeley, 1996). For phytoplasma genomes, it has been well known that the base frequencies are not equal (G+C contents is only 23.0 to 26.2 mol %). In this study, secondary structure analysis indicated that substitution rates vary with different regions of the 16S rRNA genes of phytoplasmas. Therefore, the bias of the transition : transversion ratio and different base frequencies as well as the different mutation rates along DNA sequences should be considered in phylogenetic analyses of phytoplasmas.

6.3.3 Molecular evolution of phytoplasmas

Since the phylogenetic tree constructed in this study was supported by high confidence values, it may reflect the actual phylogeny of phytoplasmas. Therefore, some inferences may be made from the phylogenetic tree. Phytoplasmas are closely related to acholeplasmas (Lim and Sears, 1992; Gundersen *et al.*, 1994) and are believed to arise from the acholeplasma-anaeroplasma-phytoplasma (AAP) branch and are much younger than mycoplasmas in evolutionary development (Maniloff, 2000). The phylogenetic tree in this study suggested that phytoplasmas might be evolved into two branches as a result.

One is the AS branch consisting of the AY group and the STOL group. Another branch is the WB group including the rest of the groups. A recent study indicated that phytoplasma genome sizes range from 530 to 1350 kb and that the genome sizes of phytoplasmas in the AY and STOL groups were on average 230 kb larger than those in all other groups (Marcone *et al.*, 1999). Therefore, phytoplasmas of the WB branch has likely suffered more drastic genome deletions than those of the AS branch during their evolutionary courses, but both branches have evolved independently.

Insect vectors are not only a natural host of phytoplasmas, but also may play an important role in phytoplasma evolution. Phytoplasmas are transmitted from plants to plants by insect vectors. When the annual plants, susceptible to a phytoplasma, are harvested or not available, the insect vectors carrying the phytoplasma have to migrate to other plants and may be forced to feed on nonhost plants such as perennial plants and trees (Hiruki, personal communication), in which the phytoplasmas likely suffer from limited energy supply. To adapt to the new niche, certain noncoding DNA fragments or introns may be deleted from the phytoplasma genome, as economization of DNA content is the characteristic property of all prokaryotes. Once the phytoplasma survives in its new ecological niche, a new group of the phytoplasma will develop and a new biological and ecological cycle will be established. This may explain why most phytoplasmas associated with diseases in perennial plants such as trees and shrubs have relatively smaller genome sizes and belong to the WB branch. For example, the genome sizes of phytoplasmas in the AP group are less than 700 kb, and ash yellows phytoplasma genome is 620 kb.

Another possibility for the development of a different major group of phytoplasma is a situation where the phytoplasma was transported by insect vectors or by infected plants from its original environment to a new environment where the host plants are unavailable. A mechanism similar to the development of tree phytoplasmas may be followed. Thus, a new phytoplasma may develop in a plant species where a new ecological niche for the phytoplasma could be established.

On the other hand, phytoplasma-affected perennial plants may serve as sources of phytoplasma infection in annual plant species via insect transmission. Annual plants are normally available in large populations during a growing season. They create excellent conditions for phytoplasmas to increase their populations, providing ample opportunities for mutations of phytoplasmas and heterogeneity of phytoplasmas in the group. However, since the host plants (perennial) are available, the phytoplasmas in perennial plants do not have high surviving pressure that gives rise to drastic mutation. Therefore, under such circumstances a phytoplasma population may be genetically stable and may have little chance to develop a new phytoplasma.

A given plant may be infected by more than one phytoplasma from different subgroups or major groups (Alma *et al.*, 1996; Lee *et al.*, 1994). Mixed infections may also occur in insect vectors. When these phytoplasmas share a common host pool, it provides ample opportunities for phytoplasmas to exchange their genetic information. As a result, a wide diversity of phytoplasmas may develop in groups such as the AY group. Another possibility is that an insect vector carrying phytoplasma A feeds on the plant infected by phytoplasma B, and thus acquires phytoplasma B. Even if the insect is not a host of phytoplasma B, genetic information from phytoplasma B may flow into phytoplasma A in the insect vector resulting in the development of a new phytoplasma.

The relationship between geographic distribution and the evolution of phytoplasmas may be inferred from the phylogenetic tree. Most phytoplasmas in the AP group were found in Europe (Seemüller *et al.*, 1998). On the basis of the phylogenetic tree, the phytoplasmas, except for the AY group and the STOL group, likely arose from phytoplasmas in the AP group and were distributed worldwide by the mechanisms hypothesized above. Most phytoplasmas in the CP group were found in North America while the phytoplasmas in the WX group were found in Europe and North America (Seemüller *et al.*, 1998). It is possible that phytoplasmas in the CP group likely evolved from phytoplasmas in the AP group in Europe with the phytoplasmas in the WX group serving as a possible bridge.

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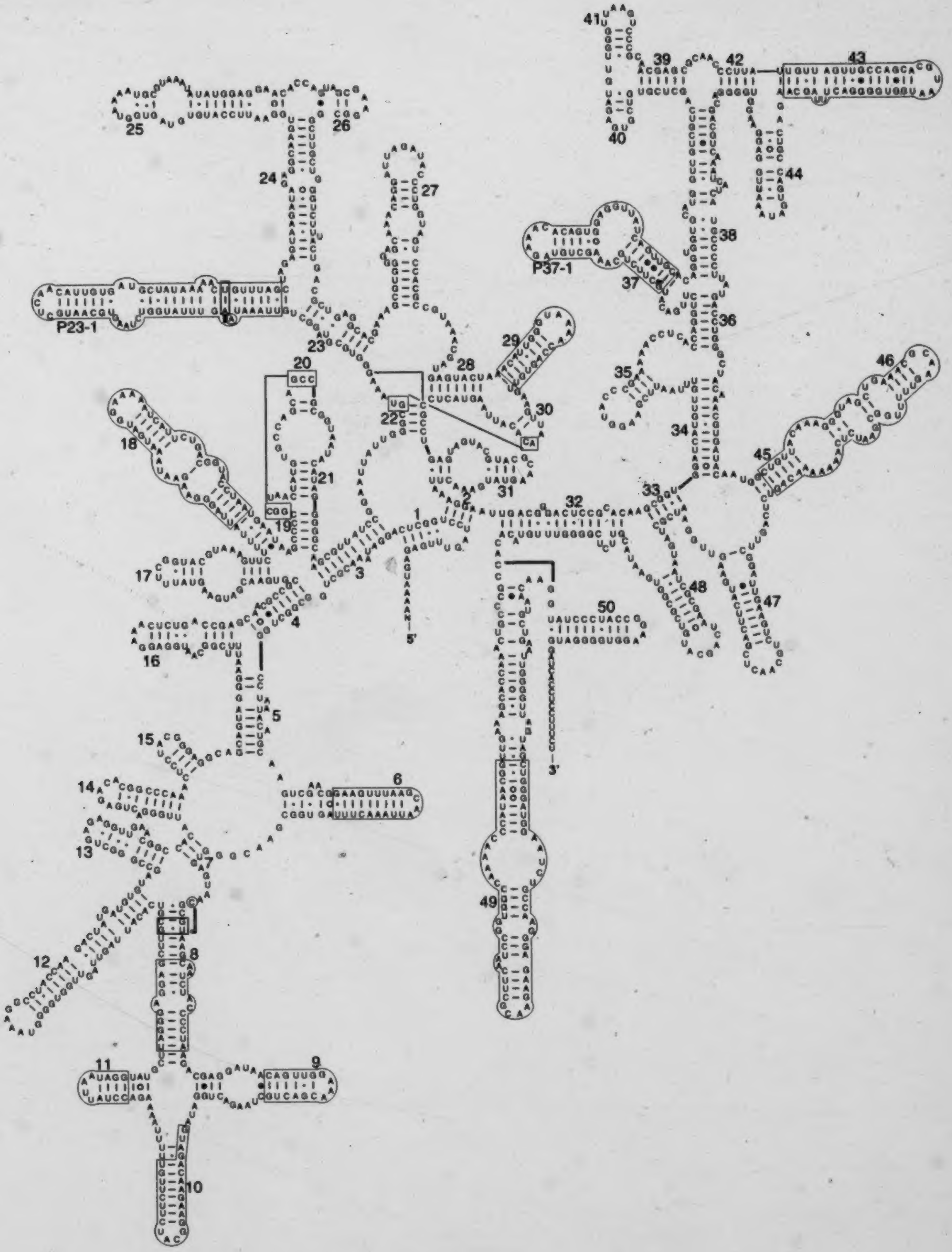
Fig. 6-1. The secondary structure model of the 16S rRNA gene of AY27 phytoplasma. The boxed regions are highly variable among phytoplasmas. The symbols used in this model are: G-C, canonical base pair (A-U, G-C); G·U, G-U base pair; G∘A, G-A base pair; U•U, non-canonical base pair.


P23-1
P37-1

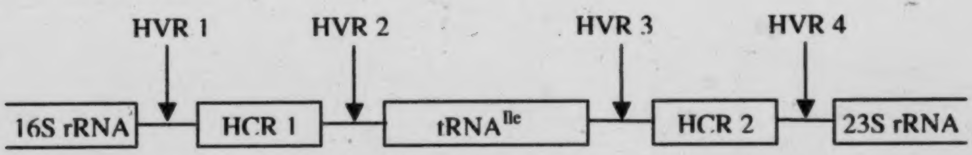
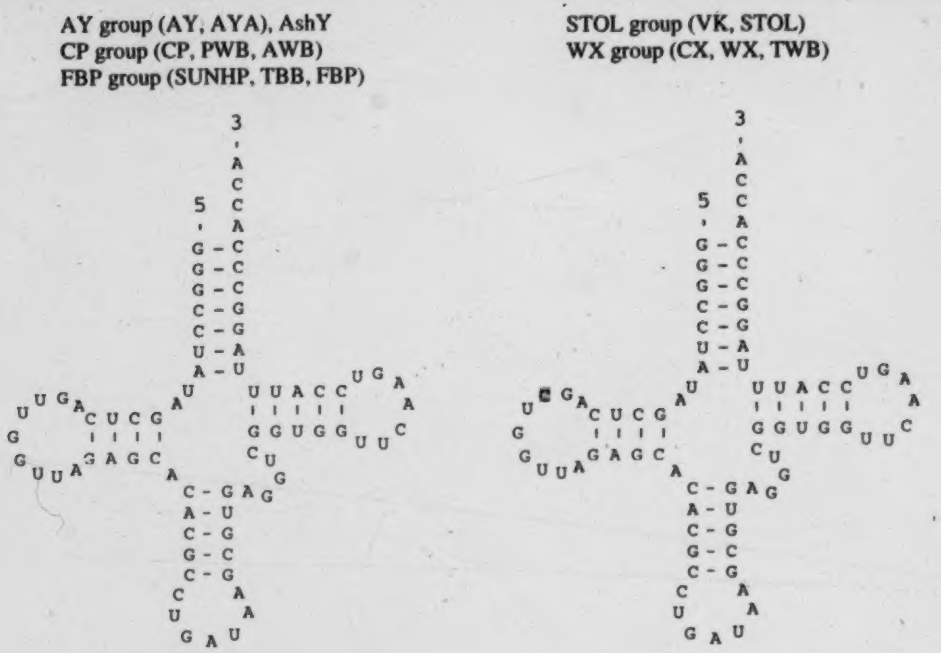
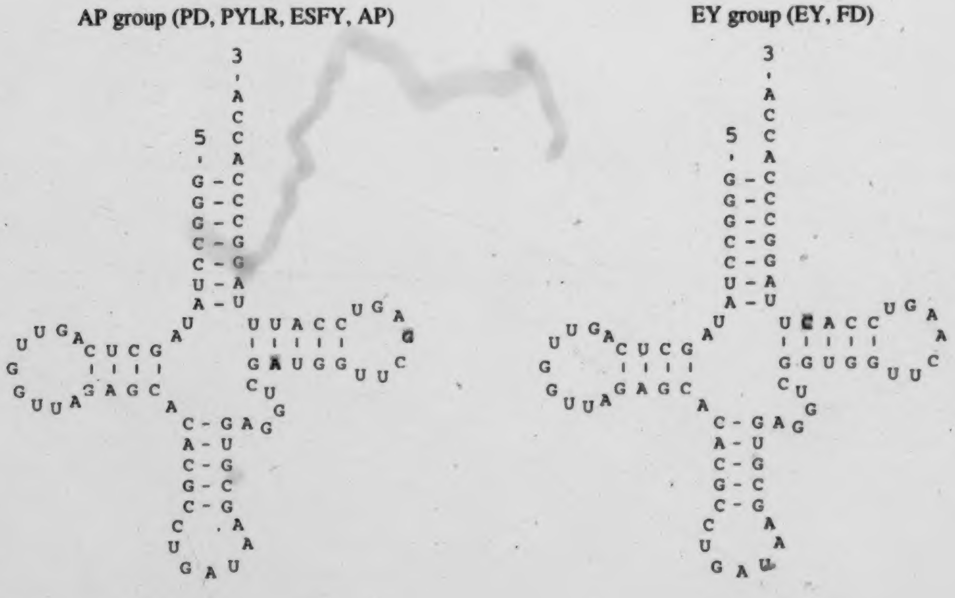


Fig. 6-2. The general structure of the 16/23S spacer region of phytoplasmas. It includes one tRNA^{Ile} gene, two highly-conserved regions (HCRs), and four highly variable regions (HVRs). The nucleotide sequences of HCR 1 and HCR 2 are as follows: 5'-TCATCTTCAGTTTTGAA(or G)AGACTTA-3' and 5'-TCTTTGAAAAGATAAA-3' respectively.



AY group (AY, AYA), AshY
CP group (CP, PWB, AWB)
FBP group (SUNHP, TBB, FBP)

STOL group (VK, STOL)
WX group (CX, WX, TWB)

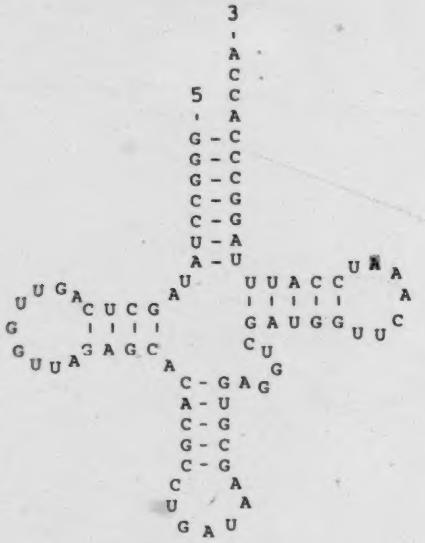


AP group (PD, PYLR, ESFY, AP)

EY group (EY, FD)

Fig. 6-3. Secondary structure of tRNA$^{\mathrm{Ile}}$ gene of phytoplasmas. The substituted bases comparing with AY phytoplasmas are shaded.

FBP group (PEP)



SCWL group (BVK) **SCWL group (RYD)**

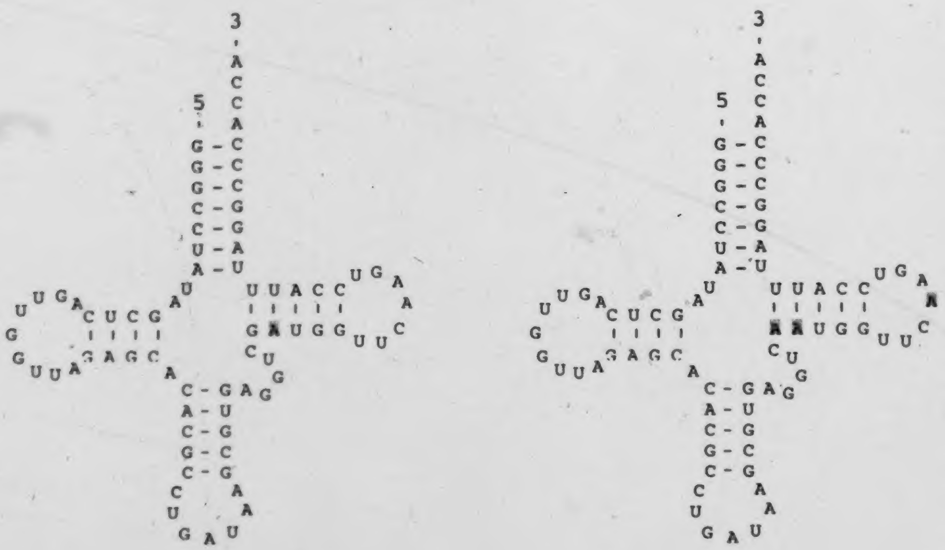


Fig. 6-3 (continue). Secondary structure of tRNA^{Ile} gene of phytoplasmas. The substituted bases comparing with AY phytoplasmas are shaded.

	AY	AYA	STOL	VK	BVK	RYD	BLL	CP	ASHY	EY	FD	EVY	CX	WX	TWB	TBB	SUNH	FBP	PEP	PD	PYLR	AT	ESFY	Alaid
AY																								
AYA	0.0111																							
STOL	0.0381	0.0374																						
VK	0.0388	0.0382	0.0007																					
BVK	0.0989	0.0998	0.1066	0.1073																				
RYD	0.1089	0.1081	0.1100	0.1109	0.0196																			
BLL	0.1129	0.1154	0.1239	0.1247	0.0557	0.0507																		
CP	0.1121	0.1129	0.1230	0.1238	0.0587	0.0538	0.0139																	
ASHY	0.1119	0.1119	0.1156	0.1164	0.0587	0.0539	0.0317	0.0352																
EY	0.1091	0.1100	0.1225	0.1233	0.0588	0.0564	0.0317	0.0324	0.0404															
FD	0.1142	0.1151	0.1269	0.1277	0.0604	0.0589	0.0354	0.0360	0.0427	0.0048														
EVY	0.1223	0.1182	0.1227	0.1235	0.0586	0.0579	0.0720	0.0726	0.0704	0.0681	0.0697													
CX	0.1030	0.1014	0.1128	0.1136	0.0540	0.0571	0.0659	0.0673	0.0742	0.0666	0.0706	0.0618												
WX	0.1053	0.1038	0.1153	0.1160	0.0563	0.0595	0.0682	0.0696	0.0765	0.0690	0.0729	0.0641	0.0021											
TWB	0.1127	0.1101	0.1155	0.1164	0.0603	0.0675	0.0708	0.0731	0.0798	0.0741	0.0775	0.0696	0.0180	0.0202										
TBB	0.1171	0.1165	0.1219	0.1218	0.0930	0.0939	0.1126	0.1125	0.1101	0.1164	0.1193	0.1009	0.0872	0.0895	0.0925									
SUNH	0.1210	0.1196	0.1259	0.1267	0.0928	0.0946	0.1125	0.1130	0.1107	0.1170	0.1198	0.1014	0.0894	0.0917	0.0948	0.0090								
FBP	0.1153	0.1155	0.1167	0.1175	0.0967	0.0944	0.1132	0.1131	0.1108	0.1171	0.1191	0.1030	0.0910	0.0934	0.0922	0.0174	0.0196							
PEP	0.1169	0.1171	0.1184	0.1191	0.0921	0.0928	0.1092	0.1090	0.1075	0.1129	0.1149	0.0975	0.0871	0.0895	0.0855	0.0189	0.0224	0.0217						
PD	0.0882	0.0875	0.0886	0.0894	0.0863	0.0839	0.0961	0.0993	0.0932	0.0936	0.0946	0.0981	0.0977	0.1001	0.0998	0.1138	0.1138	0.1128	0.1103					
PYLR	0.0927	0.0896	0.0915	0.0923	0.0908	0.0888	0.1007	0.1038	0.0969	0.0973	0.0983	0.1010	0.1022	0.1046	0.1029	0.1193	0.1200	0.1182	0.1157	0.0056				
AT	0.0905	0.0864	0.0901	0.0909	0.0862	0.0856	0.1001	0.1032	0.0986	0.0975	0.0985	0.1004	0.0992	0.1016	0.1013	0.1179	0.1187	0.1169	0.1160	0.0119	0.0126			
ESFY	0.0865	0.0841	0.0837	0.0845	0.0902	0.0847	0.0993	0.1008	0.0971	0.0968	0.0978	0.1012	0.1001	0.1024	0.1022	0.1178	0.1187	0.1169	0.1169	0.0133	0.0133	0.0133		
Alaid	0.1503	0.1485	0.1485	0.1492	0.1493	0.1492	0.1677	0.1685	0.1701	0.1648	0.1686	0.1633	0.1581	0.1607	0.1671	0.1728	0.1752	0.1742	0.1708	0.1417	0.1424	0.1442	0.1416	

Table 6-1. DNA distance matrix of the 16S rRNA gene sequence of phytoplasmas on the basis of calculation derived from the HKY85 model.

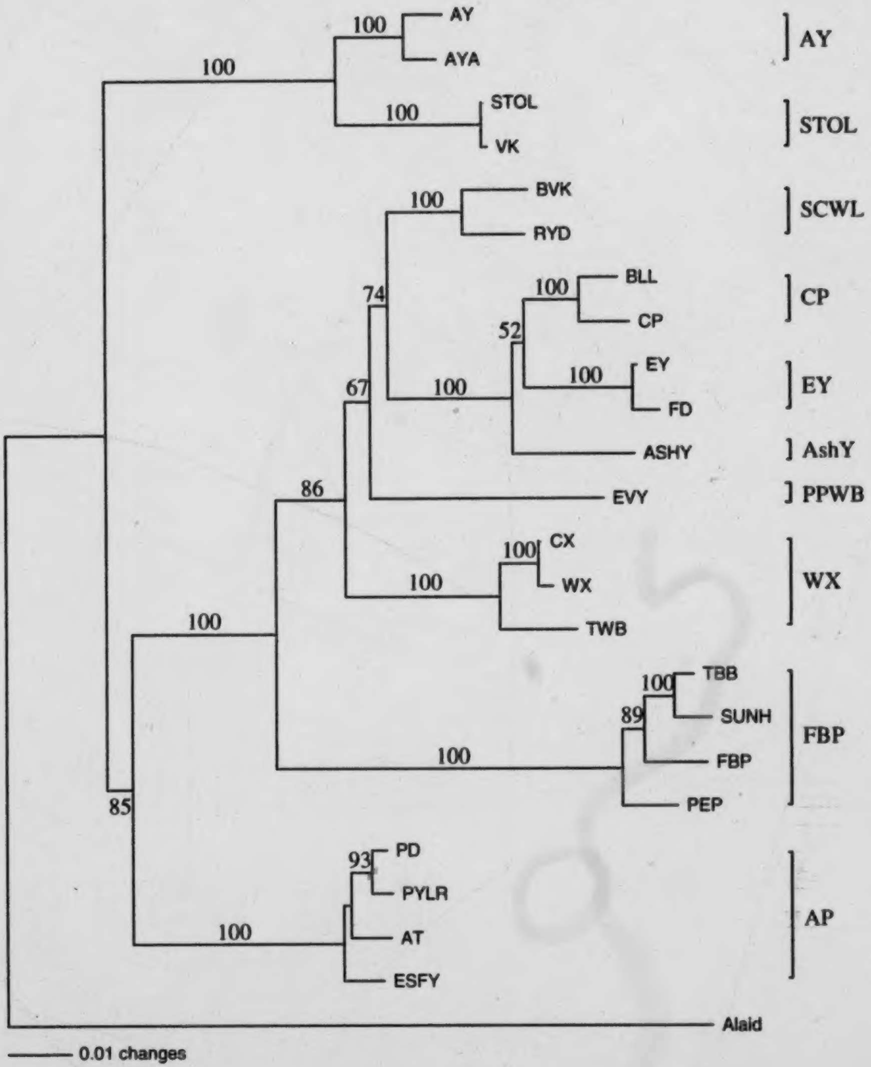


Fig. 6-4. Phylogenetic tree constructed by analysis of 16S rRNA gene sequences using neighbor-joining method. The numbers are the estimated confidence levels in percentages for the positions of the branches, determined by bootstrap analysis with 1,000 replicates. The scale bar represents sequence divergence. Abbreviations are the same as described in Table 3-1.

VII

General Discussion

7.1 Use of HMA for differentiation of phytoplasmas

HMA provides an ideal means to differentiate closely-related phytoplasmas. It was capable of detecting a single base-pair deletion/insertion or a two-base-pair substitution that was artificially introduced into a 529 bp DNA fragment derived from 16S rDNA of AY27 phytoplasma (Chapter 2). The mutations of three base pairs in the 16/23S spacer region of AWB, CP and PWB phytoplasmas in the CP group were also detected by HMA (Chapter 4), but not by RFLP, a method that has been widely used in the last decade to differentiate and identify phytoplasmas.

The sensitivity of HMA in detecting minor nucleotide differences between phytoplasma isolates can be increased by using different standard phytoplasma strains as references. For example, the phytoplasma isolates AYA showed HMA patterns very similar to the phytoplasmas in subgroup 16Sr I-B when AY27 was used as a reference. However, the differences between AYA and others were clearly observed by HMA when CP was used as a reference (Chapter 5). In addition, the single base-pair substitution between the very closely-related nucleotide sequences A and B, not detected by HMA when A or B was used as a reference, was differentiated by HMA when nucleotide sequence C was used as a reference (Chapter 2). Therefore, two or more references should be used when one tries to differentiate the phytoplasmas in the same group or to detect the minor mutations of phytoplasmas.

Substitution mutations can be differentiated from insertion/deletion mutations by HMA when the same number of nucleotides is changed, since the heteroduplexes containing gaps showed greater retardation than the heteroduplexes containing mismatches. When the heteroduplexes contained the same number of base differences, the migration of heteroduplexes containing both mismatches and gaps was faster than those containing only mismatches but slower than those containing gaps alone (Chapter 2). The reason is that the mismatches will produce a bubble if both mismatched bases are retained within the double helix, or a bulge if both bases are rotated out of the helix. Either a bubble or a bulge is unlikely to change sufficiently the conformation of the molecule to alter its electrophoretic mobility (Ganguly *et al.*, 1993). A bend in the helix will be produced when asymmetrical displacement of the two bases occurs, i.e. one base is retained in the double helix and the other base is rotated out. However, using NMR (nuclear magnetic resonance) spectroscopy, a single extra base was found to be stacked into the helix (Woodson and Crothers, 1988; Roy *et al.*, 1987; van den Hoogen *et al.*, 1988; Kalnik *et al.*, 1989). When the extra base remained within the double helix, a bend of approximately 20° was detected by NMR (Woodson and Crothers, 1988). The degree of bending caused by an extra base requires more bases in mismatches to produce the same effect (Bhattacharyya and Lilley, 1989; Wang and Griffith, 1991). Therefore, for the same number of nucleotides, gaps produce a greater degree of bending than mismatches and cause greater retardation than mismatches.

The different base compositions in the substitution or insertion/deletion mutations with the same number of base change at a gene locus can be detected by HMA. For substitution mutations, the heteroduplex containing different base compositions in mismatches had different degrees of electrophoretic retardation. For example, both mutants L5-10 and L2-2 had 7 base substitutions but different compositions compared with mutant L1-10. However, they showed much different retardation caused by heteroduplexes (Chapter 2, Fig. 1, lane 15, 17). Thus, HMA provides an important means to detect any mutations of phytoplasmas.

HMA also has been used as a tool to detect unique sequences associated with sample phenotypes of interest and subsequent derivation of full-length target gene sequences in other microorganisms (Oldach *et al.*, 2000). This approach of HMA-guided sequence analysis is potentially useful for identifying specific sequences within complex assemblages or among nonculturable organisms such as phytoplasmas.

Since HMA is highly sensitive in detecting minor differences not only in nucleotides but also in base compositions of DNA fragments, it is a powerful tool for differentiating closely-related phytoplasmas, in detecting DNA sequence divergence and sequence signatures of a given phytoplasma, and for providing useful information in the study of phytoplasma disease epidemiology and potential disease control.

7.2 Use of HMA for Classification of phytoplasmas

In the last decade, RFLP analysis of the highly-conserved genes has been widely used for the classification of phytoplasmas. However, the principle of RFLP is based on the recognition of the presence or absence of the restriction sequences in DNA fragments and many restriction endonucleases have to be used in the analysis. Consequently, RFLP analysis may erroneously increase genetic distances of the closely-related strains or reduce the significant differences of the distinct strains. DNA sequence analyses of the 16S rDNA of more than 20 phytoplasma isolates in previous studies have indicated that the RFLP-based classification of phytoplasmas in certain groups does not fully coincide with the phylogenetic relationships of the organisms (Seemüller *et al.*, 1994; Kuske and Kirkpatrick, 1992). Therefore, different RFLP patterns do not always indicate significant phylogenetic distances.

HMA is an ideal means for the identification and classification of phytoplasmas. The principle of HMA is that DNA heteroduplexes formed between different single nucleotide strands have a reduced mobility that is proportional to their degree of divergence in polyacrylamide gels under nondenaturing conditions. It is possible to measure DNA distances between two DNA fragments by calculating the mobilities of DNA heteroduplexes: $M = 0.1769-0.2346*\log D$, where M is the mobility of the DNA heteroduplex, and D is the distance between two DNA fragments in percentage (Chapter 3). In addition, HMA is capable of differentiating the closely-related strains having a level of sequence homology as high as 99.8% when suitable references are used (Chapter 3). The Working Team on Phytoplasmas of the International Research Program of Comparative Mycoplasmology of the International Organization for Mycoplasmology proposed that a threshold of a 2.5% difference in the 16S rDNA sequence homology be used for establishing a new taxonomic group (ICSB Subcommittee on the Taxonomy of *Mollicutes*. 1997). A lower threshold of a 1.2% difference was used for the finer differentiation of phytoplasmas in the AY group (Seemüller *et al.*, 1998). Since HMA is not only capable of detecting single-base differences, but also measuring genetic distance between two DNA fragments (as shown in Chapter 3), it is safe to conclude that HMA is a powerful method for the classification of phytoplasmas.

The use of HMA convincingly accomplished the classification of 24 representative phytoplasma strains into ten major groups by analyzing the partial 16S rRNA gene (1.2 kb), the full 16S rRNA gene (1.5 kb), the 16/23S spacer region (about 300 bp) and the large DNA fragment (about 1.8 kb) composing the full 16S rRNA and 16/23S spacer region. HMA analysis of all these DNA fragments for the classification of phytoplasmas was fully in agreement with the classification based on the nucleotide sequence data (Chapter 3, 6). More genetic diversity was observed among the members (SUNH, TBB, FBP) in the FBP group when the full 16S rRNA gene was analyzed by HMA. Similar use of HMA for classification of phytoplasma isolates was also reported in previous studies in which nine phytoplasmas collected from Asia, North America and Europe were classified into six groups (Zhong and Hiruki, 1994), and in which sixteen phytoplasma isolates including the field samples were classified into two major groups (Ceranic-Zagorac and Hiruki, 1996). HMA has also been employed for classification of other microorganisms such as viruses, fungi etc. (Delwart, *et al.*, 1993, 1994; Lin *et al.*, 2000; Nelson *et al.*, 1997).

HMA is more accurate than RFLP for the classification of phytoplasmas since HMA is based on the divergence of DNA sequences instead of a few of restriction sites. This permits differentiation of closely-related phytoplasmas at finer levels (Chapter 4). For example, phytoplasma isolates of AWB, CP and PWB showed identical RFLP patterns even when 15 endonucleases were used in RFLP analysis as indicated in Chapter 4 and previous studies (Lee *et al.*, 1993; Khadhair and Hiruki, 1995). In contrast, HMA profiles of both the 16S rRNA gene and the 16/23S spacer region clearly showed that AWB, CP and PWB phytoplasmas were distinguishable and can be classified into two subgroups. This conclusion was confirmed by sequence analysis of 16/23S spacer region in which two base-pair substitutions and one base-pair insertion/deletion were found between two subgroups (Chapter 4).

HMA is a simple but efficient method for the classification of phytoplasmas. When the proper reference phytoplasmas are used, a large number of phytoplasma isolates can be tested in a short time with less cost compared with RFLP analysis. Sixty-two phytoplasma isolates were clearly classified into five major groups including the AY, CP, EY, FBP, and WX groups by HMA analysis of the 16/23 spacer region (Chapter 5). Forty-eight phytoplasmas newly-collected from the field in Alberta were identified on the basis of molecular genetic data. A close molecular genetic relationship was found among JWB phytoplasma isolates collected from China, Japan and Korea and they were assigned to the EY group, which was consistent with the results derived from sequence analysis of the 16S rRNA gene. However, PaWB phytoplasma isolates from China, Japan and Korea showed genetic divergence in the16/23 spacer region and were determined to be in different subgroups in the AY group, while analysis of the 16S rRNA sequence indicated that they are closely related to each other (Wang 1997). In the AY group, four subgroups were identified among 48 phytoplasma isolates. All phytoplasma isolates in the AY group in Canada belong to subgroup I-A and I-B, except for one isolate (DAN2) from dandelion yellows disease which was identified as a unique phytoplasma and assigned to a new subgroup in the AY group. This result was supported by sequence analysis of 16S rRNA gene (Wang and Hiruki, 2001).

7.3 Selection of a suitable gene for classification of phytoplasmas

Selection of a suitable gene is very important for proper use of HMA for classification of phytoplasmas. The ribosomal rRNA genes are perhaps the best characterized mollicute genes. The organization of the mollicute rRNA genes generally follows the characteristic order 5' – 16S – 23S – 5S – 3', and function as an operon (Razin, 1985, Glaser *et al.*, 1992). Mollicutes carry only one or two sets of rRNA genes in their genomes, whereas phytoplasmas have two sets of rRNA operons called rrnA and rrnB, resembling their phylogenetic relatives, the acholeplasmas, in this respect (Schneider and Seemüller, 1994). In addition, a single $\mathrm{tRNA}^{\mathrm{Ile}}$ was found in the intergenic region between the 16S and 23S rRNA genes (Kirkpatrick and Smart, 1995, Smart *et al.*, 1996). Sequences of these highly-conserved genes serve as the most important phylogenetic, taxonomic and diagnostic tools. On the basis of the criteria for phylogenetically useful genes (Woese, 1987), comparative studies showed that 16S rRNA was the most useful phylogenetic tool (Lane *et al.*, 1985; Pechman and Woese, 1972; Sankoff, *et al.*, 1973). Currently, several genes such as a partial or full 16S rRNA gene, the 16/23S spacer region, and the large DNA fragment comprising the full 16S rRNA gene and the spacer region have been widely used as molecular tools for identification and classification of phytoplasmas (Lee *et al.*, 1993; Seemüller *et al.*, 1998; Kirkpatrick *et al.*, 1994; Marcone *et al.*, 2000). However, one must remember that the highly-conserved nature of the 16S rRNA gene may hinder a simple molecular approach as 16S rRNA sequence analysis failed to distinguish some bacterial strains, while conventional methods such as DNA homology and phenotypic characters were readily applicable (Fox *et al.*, 1992; Fraser *et al.*, 1995).

The 16/23S spacer region was found to be a suitable gene for HMA for classification of phytoplasmas in this study. The spacer region contains a highly-conserved tRNA^{Ile} gene, which may serve as a measurement of deep genealogical events, and highly variable regions that may serve as a measurement of more recent genealogical events. In addition, the spacer region is relatively small and easily separated in polyacrylamide gels. The classification by HMA analysis of the 16/23S spacer region of twenty-four representative phytoplasmas fully agrees with those derived from sequence analysis of the 16S rRNA (Chapter 3). In addition, analyses of 16/23S spacer region by HMA have revealed more variations among members of a given group and resolved more distinct subgroups (Chapter 5) than analyses of the 16S rRNA gene (Lee *et al.*, 1993; Gundersen *et al.*, 1994, 1996; Wang, 1997). Since the 16/23S spacer region is located between the highly-conserved 16S and 23S rRNA genes, it can be amplified from a broad range of phytoplasmas using a single set of universal primers, which makes it possible to analyze the organisms at the same level (Chapter 3, 5). Other less-conserved genes, such as ribosome protein genes, or the elongation factor (*tuf*) gene, used for the finer differentiation of phytoplasmas (Gundersen *et al.*, 1996; Kirkpatrick *et al.*, 1994; Marcone *et al.*, 2000; Schneider *et al.*, 1997; Smart *et al.*, 1994, 1996) were not included in this study.

7.4 Use of HMA for genetic diversity study of phytoplasmas

HMA analysis of the 16S rRNA gene and the 16/23S spacer region revealed that phytoplasmas are more diverse than previously thought based on RFLP analysis. More genetic diversity of phytoplasmas was observed among the members of the CP group (Chapter 4) by HMA than by RFLP analysis which has been extensively used for differentiation and characterization of phytoplasmas in the last decade. HMA also revealed the sequence diversity of 16/23S spacer region for PaWB phytoplasmas from different countries (Chapter 5). Therefore, the high sensitivity of HMA makes it not only a powerful tool for detecting sequence mutations, but also a useful method for studying genetic diversity and molecular epidemiology.

HMA analysis of 16/23S spacer regions from more than 50 phytoplasmas collected in Canada indicated that AY phytoplasmas are the most prevalent in Canada, followed by CP phytoplasmas (Chapter 5). Almost all phytoplasmas in the AY group belong to subgroups 16Sr I-A and B, while the phytoplasmas in subgroup 16Sr I-A are more common than phytoplasmas in subgroup 16Sr I-B in Canada. The subgroup 16Sr I-A phytoplasmas have been found only in North America while phytoplasmas in subgroup 16Sr I-B are distributed worldwide (Lee *et al.*, 1998; Seemüller *et al.*, 1998). The geographical isolation of the phytoplasmas may be correlated with the distribution of their insect vectors that are native to a particular region. The phytoplasmas in subgroup 16Sr I-A are transmitted only by *Macrosteles fascifrons* which is native to North America, while the phytoplasmas in subgroup 16Sr I-B can be transmitted by both European species *M. laevis* and North American species *M. fascifrons* (Brcák, 1979; Nielson, 1979). This may be the reason why the phytoplasmas in both subgroups 16Sr I-A and B have been found in North America, while only phytoplasmas in subgroup 16Sr I-B have been found in Europe except for one rare example associated with gladiolus virescence (Vibio *et al.*, 1994).

Recognition of ecological diversity of phytoplasma strains in nature may provide a way to study the epidemiology of phytoplasma-induced diseases, and to prevent the potential spread of phytoplasma diseases due to international germplasm exchange. Information on genetic diversity within a phytoplasma strain is not only necessary for the development of disease-resistant lines, but also provides a tool to study the evolution of new strains in a given field.

Similar symptoms can be induced by distantly-related phytoplasmas, and different sets of symptoms can be induced by closely-related phytoplasmas as indicated in Chapter 5 and previous studies (Gibb *et al.*, 1996; Marcone *et al.*, 1996a). PaWB phytoplasma in Japan was identified to be the member of subgroup 16Sr I-B while PaWB isolates in China and Korea were assigned to subgroup 16Sr I-D on the basis of HMA analysis of the 16/23S spacer region. On the other hand, at least 29 or 15 plant species were infected by very closely-related phytoplasmas in subgroup 16Sr I-A or B, respectively, resulting in very similar yellows symptoms in Alberta. Since the natural hosts of phytoplasmas are plants and insects, the host specificity of a given phytoplasma must be related to the pathogenicity genes and insect transmission genes, the host preference of insect vector feeding on plants, and the geographic distribution of insects. Presumably, subgroups with distinct gene pools have become discrete through evolution after isolation from an ancestral population. This may have resulted from the course of repeated phytoplasma multiplication in different plant or insect hosts, or from geographic dispersal of ancestral hosts which are controlled by different sets of environmental conditions.

7.5 The limitations and conditions for HMA

Although HMA is a powerful means for differentiating, identifying, classifying and studying the genetic diversity of phytoplasmas, it has certain limitations. It cannot be applied to analyze the whole genomic DNA or very large DNA fragments (more than 2-3 kb) since the large DNA homoduplexes and heteroduplexes will migrate very slowly in a polyacrylamide gel, making them difficult to separate clearly. In addition, HMA is not suitable for differentiation of microorganisms with more than 35% genetic divergence since all the heteroduplexes will migrate very slowly and thus will not be easily differentiated between different strains. According to the guidelines set by the International Committee on Systematic Bacteriology, bacterial strains in a species should share at least 70% DNA homology on the basis of a complete sequence of the bacterial genome (Wayne *et al.*, 1987). Thus, HMA is most suitable for distinguishing different strains at the species level or lower.

Certain experimental conditions must be met for the successful use of HMA. It has to be used with PCR to obtain sufficient DNA fragments selected from a certain region in genomic DNA. Nonspecific DNA fragments should be eliminated from PCR products by improving the quality of DNA extracted from phytoplasma-infected samples, by changing the PCR protocols or by adjusting PCR conditions. Otherwise, they will confuse identification of the target heteroduplex bands and may produce extra heteroduplexes that will lead to incorrect conclusions. For identification of an unknown phytoplasma isolate, suitable known phytoplasma strains have to be used as references, as shown in Chapter 5. For classification of other microorganisms, a suitable gene has to be selected as a reference as was done for the classification of phytoplasmas (Chapter 3).

7.6 Molecular evolution of phytoplasmas

The phylogenetic trees were constructed on the basis of analyzing the 16S rRNA gene and 16/23S spacer region sequences of phytoplasmas by using neighbor-joining method (Chapter 6). Ten distinct major groups were established among 24 phytoplasma strains on the basis of branch patterns and sequence differences of at least 2.5%. The confidence level for each group is higher than 95%. The results fully agree with previous studies except for the PEP phytoplasma strain (Seemüller *et al.*, 1998). In this study, on the basis of confidence level and the length of the branch, PEP was classified into the FBP group instead of an independent group. The sequence difference between PEP and the members of FBP group is 2.4% which is also lower than the threshold of 2.5% for assignment of phytoplasmas into different groups set by the Working Team on Phytoplasmas of the International Research Program of Comparative Mycoplasmology of the International Organization for Mycoplasmology (1994, 1996). Therefore, PEP phytoplasma should be assigned in a different subgroup within the FBP group.

The evolutionary history of phytoplasmas could be inferred from the phylogenetic tree. Two branches, the AS branch including the AY and STOL group, and the WB branch including all other groups, might have evolved from their ancestral acholeplasma. The phytoplasmas in the WB branch may suffer from more deletions of their genomes than those in the AS branch since the genome sizes of phytoplasmas in the WB branch were, on average, 230 kb less than those in the AS branch (Marcone *et al.*, 1999). The members of the AY and STOL groups might have undergone substitution and/or deletion mutations so as to form different subgroups. In addition, the AY group consisting of 14 subgroups is the largest one in the classification of phytoplasmas. AY phytoplasmas have an extremely wide host range. Among more than 250 phytoplasma isolates identified so far, nearly half of them belong to the AY group, which might be associated with the high mutation rate of AY phytoplasmas. It is anticipated that continuous genetic evolution takes place for phytoplasmas to adapt to varied host-parasite interactions under different environmental conditions and progressive agriculture and forestry practices.

The drastic reduction in genome size might be related to genetic drift that can occur during the course of continuous phytoplasma evolution. When phytoplasmas were transported to a new environment where the host plants and/or host insect vectors were not available, or when the host plants suddenly were not available in the original environment, insect vectors were forced to feed on non-host plants and phytoplasmas suffered from starvation. To survive in new environment, phytoplasmas had to use limited energy efficiently by deleting non-informational DNA such as introns, other noncoding sequences, and even some coding sequences whose translated organic molecules could be supplied by both host plants and insect vectors. This lack of certain genomic information may also explain why phytoplasmas cannot be cultured in an artificial medium. In addition, phytoplasmas with a small genome may replicate efficiently and may tolerate a high mutation rate. However, the phytoplasma genome sizes observed today may be at the point where further reductions in genome size are not possible.

The secondary model of the 16S rRNA gene of phytoplasmas has been established in Chapter 6. A total of ten major variable sections have been identified for all phytoplasmas examined in this study, although a few substitutions are scattered in other areas. A compensatory mutation in the complementary position to stabilize the actual stem was observed in most cases, although the general alternative to noncanonical base pairing was found in Chapter 6. Compared to the 16S rRNA, the tRNA isoleucine (Ile) located in the spacer region between the 16S and 23S rRNA gene was highly conserved during the course of evolution, suggesting that the rRNA^{Ile} evolved very slowly. The two nucleotide sequences located downstream and upstream of the tRNA^{Ile} gene were also almost identical across all phytoplasmas, suggesting that they may be involved in the processing of rRNA genes and tRNA gene or other functions during rRNA transcription.

While genetic drift may result in the formation of new groups of phytoplasmas, the effects of gene flow may produce new subgroups in the same major group. There are two possibilities for gene flow of phytoplasmas. One is that the gene flow occurs in the host plants. In nature, a given plant can be infected by more than one phytoplasma (Alma *et al.*, 1996; Lee *et al.*, 1994). Previous studies have revealed that a single plant is often infected by a predominant phytoplasma and by one or more other phytoplasmas that are present in lower titers (Alma *et al.*, 1996; Lee *et al.*, 1995; Marcone *et al.*, 1996b). Therefore, these phytoplasmas within the same group or between groups in the common pool may interact with one another and exchange their genetic information, resulting in the evolution of new strains. These new strains evolved within a given phytoplasma group or between groups may become isolated in new habitats. Hence, frequent interactions among constituent phytoplasma populations in a common pool and the isolation of new strains in new habitats may lead to the formation of a diverse phytoplasma group (e.g. AY group) that comprises many distinct subgroups (Gundersen *et al.*, 1996; Lee *et al.*, 1998).

Vectors may play a major role during the evolution of new phytoplasmas. The insect vector carrying a phytoplasma A from its original host is transported by wind or other means of transportation to a new environment where an appropriate host plant may not be available. They will be forced to feed on nonhost plants. If a plant species, which is not susceptible to phytoplasma A, is infected by phytoplasma B, phytoplasma B may be acquired by insect vectors carrying phytoplasma A, which visit that plant. These two phytoplasmas will have excellent opportunities to interact with each other and genetic information exchange may occur. One or more genes of one phytoplasma may flow into another phytoplasma, resulting in the development of a new phytoplasma strain. After insect vectors carrying a new phytoplasma migrate to a susceptible host plant, the new phytoplasma disease may be induced. Thereafter, the phytoplasma may evolve independently from the parent strain and begin to establish a new biological and ecological cycle.

A good example of possible gene flow in insect vectors may be dandelion yellows phytoplasma which was identified as a member of a new subgroup in the AY group by HMA analysis of the 16/23S spacer region (Chapter 5). Previous study also recognized dandelion yellows phytoplasma as a member of a new subgroup in the AY group on the basis of RFLP analysis of the 16S rRNA gene sequences (Wang and Hiruki, 2001). Thus, it was hypothesized that the 16S rRNA gene of dandelion yellows phytoplasma originated from AY phytoplasmas by insertion of a small DNA fragment (Wang and Hiruki, 2001). Dandelion is one of the natural plant hosts of the AY phytoplasmas in subgroup 16Sr I-A. *Macrosteles fascifrons* is known to transmit AY phytoplasmas (16Sr I-A) and CP phytoplasmas (Chiykowski, 1964; Tsai, 1979). The vector *M. fascifrons* may migrate from its original host (e.g. canola) infected with AY phytoplasma (16Sr I-A) to alfalfa plants infected with AWB phytoplasma. The vector will be forced to feed on the infected alfalfa plants. AWB phytoplasma will be acquired by the insect vector carrying AY phytoplasma. The opportunity for gene flow may occur through the exchange of genetic information in the common host pool and a new phytoplasma may develop in the insect vector. When the insect visits dandelion plants again, the visited dandelion may be infected by the newly-evolved phytoplasma as identified in the Chapter 5. To confirm this hypothesis, the genomic maps of AY and AWB phytoplasmas and a study on insect vector transmission would have to be conducted.

7.7 General Conclusions

Experimental evidence presented in this study strongly supports the conclusion that HMA is a useful method for the detection, identification, classification, and analysis of the genetic diversity and molecular epidemiology of phytoplasma diseases. HMA is based on the principle that DNA heteroduplexes formed between related DNA sequences have reduced mobilities in polyacrylamide gels under non-denaturing conditions and that the degree of their delay is proportional to the extent of specific divergence. By analyzing a set of mutants containing 1-4 base-pair substitutions and 1-3 base-pair deletions that were created by *in vitro* mutation of the PCR-amplified 16S rRNA gene, HMA was shown to be capable of detecting a single base-pair deletion/insertion or a single two-base-pair substitution in a 520 base-pair DNA fragment. A single base-pair substitution was differentiated by HMA when a suitable reference was used. Heteroduplex mobilities were affected by base number and base composition in the mismatches or gaps and were proportional to the degree of DNA divergences. Gaps caused greater retardation in heteroduplex mobility than mismatches did. HMA was also highly sensitive in detecting mixed infections of phytoplasmas.

HMA was used successfully to classify 24 representative phytoplasma strains into 10 major groups by analyzing the 16S rRNA and 16/23S spacer region. The classification of phytoplasmas by HMA fully agreed with that derived from DNA sequence analysis. HMA analysis of the 16S rRNA gene showed more diversity in the FBP group when AY27 was used as a reference. Among the partial 16S rRNA, full 16S rRNA, 16/23S spacer region, and the large DNA fragment comprising the full 16S rRNA and 16/23S spacer region, the 16/23S spacer region was demonstrated to be a suitable gene for using

HMA for classification of phytoplasmas. This research has indicated that HMA is a reliable, simple, rapid, and accurate method to detect and estimate the genetic divergence of phytoplasmas when other methods, such as RFLP analysis, are not readily applicable. It can be employed for: a) identification and classification of phytoplasmas; b) gene monitoring of a large population of phytoplasmas; c) studying the dynamic nature of phytoplasma populations of different origins; d) detection of mutations of phytoplasma genes important to pathogenesis and transmission; e) field survey of phytoplasma disease for the study of phytoplasma disease epidemiology. In addition, it is less expensive than RFLP analysis which requires many expensive endonucleases.

For the first time, two subgroups in each of the phytoplasmas AWB, CP and PWB were identified by HMA analysis, but not by RFLP analysis, of the 16S rRNA and 16/23S spacer region amplified from phytoplasma strains AWB, CP and PWB collected from the field from 1992 to 1998. However, the occurrence of phytoplasmas in two subgroups was uneven in AWB, CP and PWB diseases. The results further confirmed that HMA is more sensitive and accurate than RFLP in detecting genetic diversity and classifying phytoplasmas.

HMA analysis of the 16/23S spacer region of phytoplasmas collected in Canada, Asia, and Europe has revealed five groups and nine subgroups among 62 phytoplasma isolates using representatives in each group as references. More genetic diversity of phytoplasmas was observed than in previous studies. Twenty-six plant species were, for the first time, reported to be associated with phytoplasmas in Alberta. The prevalent phytoplasmas are AY phytoplasmas in subgroups 16Sr I-A and 16Sr I-B and more phytoplasmas were found in subgroup I-A than in subgroup I-B. The results provide very useful information for studying disease epidemiology and control.

The secondary structure model of the 16S rRNA gene of phytoplasmas was established and the highly variable sections were identified among phytoplasmas. The tRNA^{Ile} located in the 16/23S spacer region evolved slowly and was variable only in the T stem and loop for most phytoplasmas. Two highly-conserved sequences located downstream and upstream of the tRNA^{Ile} gene may be involved in the processing of rRNA genes and the tRNA^{Ile} gene. The other parts of the spacer region are highly variable because of the insertion and/or deletion of different sizes of DNA fragments resulting in the different lengths of the 16/23S spacer region. A phylogenetic tree was constructed on the basis of analyses of the 16S rRNA gene. The twenty-three phytoplasmas were classified into ten major groups with a high level of confidence for each group. The divergent phytoplasmas might evolve into two branches from their ancestor. The AS branch, comprising the AY group and STOL group, might generally suffer from less deletion of their genomes than the WB branch that includes all other phytoplasmas. The phytoplasmas in the WX group that were found in both Europe and North America likely arose from phytoplasmas in the STOL group in Europe and likely served as a bridge for the Europe-North America connection, i.e. phytoplasmas in the CP group in North America might have originated from phytoplasmas in the AP group in Europe via the WX group. Two models of phytoplasma evolution were proposed: genetic drift resulting in the development of new major groups by severe deletion of genome to adapt to a new environment, and gene flow producing new subgroups in the same major group by genetic exchange in the common pool of host plants or insect vectors.

7.8 Future Perspectives

7.8.1 Genetic mapping of phytoplasma genomes

Although phytoplasmas have been placed in the class *Mollicutes* on the basis of extensive phylogenetic analysis of conserved genes, G+C content, and genome sizes, little is known about the characteristics of phytoplasma genomes including some important genes involved in pathogenesis, vector transmission, DNA replication and repair systems. Further study of these genes will provide some insights into the biological characteristics of phytoplasmas and aid in developing strategies for phytoplasma disease management.

Recently, three physical maps of phytoplasmas have been constructed on the basis of restriction patterns of genomes with rare-cutting endonucleases. Certain genes such as conserved genes and the elongation factor gene have been located on physical maps (Firrao *et al.*, 1996; Lauer and Seemüller, 2000; Padovan *et al.*, 2000). The studies revealed that mycoplasmas and phytoplasmas shared limited similarities at the gene sequence level and there were extensive rearrangements in phytoplasma genomes. To characterize the important genes, physical maps with higher resolution and the comprehensive genetic maps of phytoplasmas should be constructed in future research. A far better resolution can be achieved if a genome is represented by a complete, ordered gene library consisting of plasmid, cosmids, λ phages or even yeast artificial chromosome or bacterial artificial chromosome. In addition, phytoplasmas in the AP, CP, and WX groups have relatively small genome sizes ranging from 600 to 700 kb (Marcone *et al.*, 1999). It will be much easier to construct a genomic library of these phytoplasmas so as to produce physical maps with high-level resolution or genetic maps. This information will be useful for the isolation and characterization of the important genes and for understanding gene arrangements.

7.8.2 Insect vector-phytoplasma interaction

Insect vectors are a natural host of phytoplasmas and play a major role in providing ecological niches for phytoplasmas and in the epidemiology of diseases. However, little is known about the molecular basis of vector-phytoplasma interactions including recognition, adhesion and penetration of host cells that define the nature of transmission specificity, and mixed phytoplasma infections in insect vectors that facilitate the mixed infection in host plants and evolution of phytoplasmas. A gene related to adhesion of mollicutes to host cells has been cloned and identified in *Spiroplasma citri* (Fletcher *et al.*, 1998). With respect to the interaction between phytoplasmas and insect vectors, an early study indicated that if a phytoplasma was maintained in periwinkle in a greenhouse for a long time, it could lose vector transmissibility in plants. This loss in transmissibility was associated with the loss of extensive rearrangement of a 4.8 kb extrachromosomal DNA (Denes and Sinha, 1992). Such information is essential to the improvement of our approach to phytoplasma disease management.

7.8.3 Mechanisms of the pathogenicity of phytoplasmas

The effects of phytoplasma infection, more precisely impairment of phloem function and the disturbance of the normal hormone balance, have been observed in the development of phytoplasma-induced plant diseases (Gabridge *et al.*, 1985). These changes in host plants as a result of phytoplasma infection can be caused by plant responses to signals triggered by infection or by the expression of phytoplasma genes. However, thus far there are no conclusively demonstrated mechanisms to explain the pathogenicity of phytoplasmas. Differential cDNA display studies revealed that the expression of differential gene including protein kinase gene might be responsible for the induction of virescence and phyllody in plants during early stages of infection with AY phytoplasmas (Smart and Kirkpatrick, 1996; He *et al.*, 1998). However, it is unknown whether these genes originated from plants or from phytoplasmas.

Promising studies (Foissac *et al.*, 1997; Jacob *et al.*, 1997) based on transposon *Tn 4001* mutagenesis have disclosed that one of the *Tn 4001* mutants of *Spiroplasma citri* did not multiply in the leafhopper vector and thus could not be transmitted to plants. Most studies on gene transfer in mollicutes have used as a selectable marker the *tet* M tetracycline resistance determinant found in *Tn 916* since mollicutes in general are sensitive to tetracycline (Dybvig and Voelker, 1996). Further study on the mechanisms of pathogenicity of phytoplasmas with the recent advances in the development of molecular biological techniques will expand our understanding of disease development and provide important information for disease control.

7.8.4 Disease-resistance genetic engineering

Traditionally, phytoplasma diseases have been managed by planting clean stocks or disease-resistant varieties combined with control of insect vectors, or by applying certain cultural practices to eliminate the sources of phytoplasmas (Nair, 1988). Among these disease management strategies, breeding of disease-resistant cultivars is the most efficient way to combat against many devastating phytoplasma diseases (Sinclair *et al.*, 1997; Thomas and Mink, 1998). Modern molecular technology has facilitated genetic engineering in plant breeding by introducing target genes into selected crops. Since some antibodies against the class *Mollicutes* inhibit their growth and metabolism *in vitro*, an engineered antibody gene against the major membrane protein of the stolbur phytoplasma was transferred into tobacco plants. Subsequently, transgenic plants showed reduced disease development (Le Gall, *et al.*, 1998).

Certain compounds such as proteinase inhibitors and lytic enzymes such as chitinase and β-1-3-glucanase produced by plants have been reported to be related to disease resistance and identified in certain phytoplasma infected plant species such as poplar and periwinkle (Davis *et al.*, 1991; Zhong and Hiruki, 1994). Introduction of these genes and other genes, such as the genes for insect resistance, into the economically important plant species by genetic engineering, will open a new era of disease management for this group of unique phytopathogens.

7.9 Reference

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